

### **REMARKS**

Claims 33-34, 33-40, 87, 89, 92, 101 and 103-108 are currently pending. Claims 33, 38-39, 87, 89, 92, 101, 103 and 104 have been amended. Claims 38, 41-52, 88, 90, 91, 93-100, 102 and 105 have been canceled without prejudice.

Claim 33 has been amended to recite the genus of compounds of previously presented claim 48, which is supported by the first embodiment presented on page 11-12 of the specification. Claims 33 and 34 have been amended to delete "*Flaviviridae*" and to recite "hepatitis C virus." Support for this amendment is found, for example, in the specification at page 36, lines 14-16.

Claim 38 has also been amended to recite "or a phosphate thereof, or a pharmaceutically acceptable salt or ester thereof." Support for this amendment is found, for example, in the specification at page 11, line 19.

Claims 38-39, 87, 89, 92, 101 and 103-104 have been amended to depend from claim 33. No new matter has been added by the amendments.

#### **I. Double Patenting Rejections.**

The Examiner has rejected claims 33-34, 37, 39-40, 48-50, 89, 100 and 105 under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claim 4 of U.S. Patent No. 7,192,936 ("the '936 patent"). Applicants respectfully disagree.

Obviousness-type double patenting is a judicially created doctrine intended to prevent improper timewise extension of the patent right by prohibiting the issuance of claims in a second patent which are not "patentably distinct" from the claims of a first patent. *See In re Braat*, 19 U.S.P.Q.2d 1289, 1291-92 (Fed. Cir. 1991). In *General Foods Corp. v. Studiengesellschaft Kohle mbH*, the Federal Circuit further explained that in an obviousness-type double patenting rejection "it is important to bear in mind that comparison can be made only with what invention is *claimed* in the earlier patent, paying careful attention to the rules of claim interpretation to determine what invention a claim *defines* and not looking to the claim for anything that happens to be mentioned in it as though it were a prior art reference." 23 U.S.P.Q.2d 1839, 1845 (Fed. Cir. 1992). Applicant respectfully submits that the Examiner is mistaken concerning what is claimed in claim 4 of the '936 patent, and by not considering what claim 4 defines, the Patent Office arrives at a legally improper double patenting rejection of the claims.

The instant claims are drawn to methods for treating a hepatitis C virus infection with certain 2'-branched nucleoside compounds that induce a specific mutation in the hepatitis C virus, in alternation or combination with a second antiviral agent that induces a different mutation. Claim 4 of the '936 patent recites a method of treating a Flaviviridae infection with a genus of 2'-trifluoromethyl substituted nucleosides, in combination with certain antiviral agents. There is no overlap between the compounds of claim 4 of the '936 patent and the compounds of the instant claims because the compounds of the instant claims are not 2'-trifluoromethyl substituted nucleosides. Furthermore, claim 4 of the '936 patent does not disclose that the second agents used in combination therapy induce mutations in a hepatitis C virus. Indeed, the claims of the '936 patent mention nothing about hepatitis C virus mutations. Therefore, reading the claims of the '936 patent, one skilled in the art would have no motivation to treat a hepatitis C virus infection with the presently claimed 2'-branched nucleosides in combination or alternation with a second mutation-inducing antiviral agent as recited in the instant claims. For these reasons, the instant claims are patentably distinct from the claims of the '936 patent. *See Braat*, 19 U.S.P.Q.2d at 1291. Therefore, Applicants request that the double patenting rejection be withdrawn.

The Examiner has rejected claims 33-34, 37, 39-40, 48-50, 89, 100 and 105 under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 15 and 18 of U.S. Patent No. 7,169,766 ("the '766 patent"). Applicants disagree.

The instant claims recite, *inter alia*, the treatment of hepatitis C virus infection with a 2'-branched pyrimidine nucleosides, while claims 15 and 18 of the '766 patent recite 2'-branched triazolo-, imidazolo- and pyrazolopyrimidine nucleosides. The instant claims further recite combination therapy with a specific class of mutation-inducing anti-hepatitis C virus agent not mentioned in the claims of the '766 patent. Thus, no overlap exists between the instant claims and the claims of the '766 patent. The Examiner has failed to demonstrate how one skilled in the art would have any motivation, based on claims 15 and 18 of the '766 patent, to treat a hepatitis C virus infection with the presently claimed compounds in combination or alternation with a second mutation-inducing antiviral agent as recited in the instant claims. For these reasons, the instant claims are patentably distinct from the claims of the '766 patent and the double patenting rejection must be withdrawn.. *See Braat*, 19 U.S.P.Q.2d at 1291.

Furthermore, the policy behind a double patenting rejection—the prevention of an unjustified extension of the term of a patent—does not support the Examiner’s rejections in this case. *See In re Braat*, 19 U.S.P.Q.2d at 1291-92; *see also In re Kaplan*, 789 F.2d 1574, 1579 (Fed. Cir. 1986) (“the basis for...obviousness-type double patenting rejections is timewise extension of the patent right”). Allowance of the instant claims would not result in the timewise extension of the terms of the ‘936 or the ‘766 patents because, as demonstrated above, the instant claims are directed to an entirely different invention than the claims of the ‘936 and ‘766 patents because no overlaps exists between these claims. Therefore, for the above-mentioned reasons, Applicants respectfully request that the double patenting rejections be withdrawn.

## **II. Rejection under 35 U.S.C. § 112, first paragraph: Written Description.**

The Examiner has rejected claims 33-34, 37, 39-40, 48-50, 89, 92 and 100-108 under 35 U.S.C. § 112, first paragraph, for alleged failure to comply with the written description requirement. Specifically, the Examiner alleges that the specification lacks written description because it does not provide specific examples of drugs that induce a mutation in a *Flaviviridae* other than the mutation recited in the instant claims. (Office Action, page 6). Applicants respectfully disagree.

To satisfy the written description requirement, a patent specification must describe the claimed invention in sufficient detail that one skilled in the art can reasonably conclude that the inventor had possession of the claimed invention. Manual of Patent Examining Procedure (“MPEP”) § 2163. Furthermore, there is a strong presumption that the claims as originally filed satisfy the written description requirement. *Id.* (citing *In re Wertheim*, 541 F.2d 257, 263, 191 U.S.P.Q. 90, 97 (C.C.P.A. 1976) On the other hand, the claimed invention may not be adequately described if the claims require an essential or critical feature which is not adequately described in the specification and which is not conventional in the art or known to one of ordinary skill in the art. *Id.*; *see also Falkner v. Inglis*, 448 F.3d 1357, 79 U.S.P.Q.2d 1001, 1006 (Fed. Cir. 2006) (Indeed, “[a] patent need not teach, and preferably omits, what is well known in the art.”) (emphasis added).

It is understood in the art of virology that viruses, such as hepatitis C, mutate to develop resistance to antiviral drugs. (*See, e.g.*, Specification at page 9, line 26 – page 10, line 10). One skilled in the art would expect that the administration of an antiviral drug will eventually induce certain mutations in a virus, resulting in resistance to the antiviral drug.

The present disclosure teaches that 2'-branched nucleoside compounds induce a specific mutation in a hepatitis C virus, and administration of a second antiviral agent that induces a different mutation in alternation or combination with a 2'-branched nucleoside compound suppresses drug resistance by viral mutation. (Specification, page 9). Therefore, one skilled in the art would understand that any anti-hepatitis C virus drug that induces a mutation other than that recited in the instant claims may be used in the instantly claimed methods. Examples of drugs that induce alternative mutations in a hepatitis C virus are known in the art. Interferons, such as type 1 interferon, which induce mutations in the RNA-activated protein kinase-binding domain of nonstructural 5A protein of the hepatitis C virus are described in M. Gale *et al.*, "Control of PKR Protein Kinase by Hepatitis C Virus Nonstructural 5A Protein: Molecular Mechanisms of Kinase Regulation," *Mol. & Cell. Bio.*, 18:9, 5208-18 (1998) ("Gale") (copy enclosed). Two hepatitis C virus protease inhibitors, VX-950 and BILN-2061, which induce different mutations in the virus, are described in Lin, Kai Lin *et al.*, "VX-950, A HCV Protease Inhibitor, Retains Potency Against BILN-2061 Resistant Replicon Cells," *Hepatology*, 38(4): 638A (2003) ("Lin") (copy enclosed). These examples demonstrate that drugs that induce mutations in a hepatitis C virus are well-known in the art. Therefore, these drugs need not be explicitly described in the specification for the specification to meet the written description requirement. *See Falkner v. Inglis*, 79 U.S.P.Q.2d at 1006. For these reasons, Applicants request that the rejection for lack of written description be withdrawn.

### **III. Rejection under 35 U.S.C. § 112, first paragraph: Enablement.**

The Examiner has rejected claims 33-34, 37, 39-40, 48-50, 89, 92 and 100-108 under 35 U.S.C. § 112, first paragraph, for alleged lack of enablement. The Examiner alleges that the specification "does not reasonably provide enablement" for methods of treating any other *Flaviviridae* virus other than hepatitis C virus. (Office Action, page 8). The Examiner admits that the specification is enabling for the treatment of hepatitis C virus infections comprising administering a 2'-branched nucleoside in combination with interferon- $\alpha$ , ribavirin, levovirin or viramidine. *Id.* However, the Examiner alleges that the specification does not provide enablement for methods of treating hepatitis C virus infections comprising administering a 2'-branched nucleoside in combination with one or more drugs that directly or indirectly induce a mutation other than a change from serine to a different amino acid in

the conserved consensus sequence, *XXSGXXT*, of domain B of the RNA polymerase region. *Id.* Applicants respectfully disagree.

Factors to be considered in determining whether a disclosure meets the enablement requirement have been set forth in *In re Wands*, 8 U.S.P.Q.2d 1400, 1404 (Fed. Cir 1988). They include (1) the nature of the invention, (2) the breadth of the claims (3) the presence or absence of working examples, (4) the amount of direction or guidance presented, (5) the state of the prior art, (6) the amount of experimentation necessary, (7) the predictability or lack thereof in the art, and (8) the level of skill in the art. The initial burden is on the Office to provide evidence of non-enablement for each of these factors. (See MPEP §§ 2164.01(a); 2164.04; *In re Wands*, 8 U.S.P.Q.2d at 1404.)

### **1. Nature of the Invention**

The claims, as amended herein, are drawn to the treatment of a hepatitis C virus infection with a 2'-branched nucleoside of a specific formula and a second drug that induces mutation other than a change from serine to a different amino acid in the conserved consensus sequence, *XXSGXXT*, of domain B of the RNA polymerase region of the virus.

### **2. The Breadth of the Claims**

The Examiner states that the breadth of the claims is "exceedingly large and fails to receive adequate support in the specification." (Office Action, page 9). Applicants respectfully point out that the claims have been amended to delete "*Flaviviridae*" and to recite "hepatitis C virus." Therefore, the breadth of the claims with respect to the virus treated is not overly broad. Furthermore, the type of drugs that may be used in combination with the specific 2'-branched nucleosides of the instant claims are limited by their ability to induce specific mutations in a hepatitis C virus. Applicants respectfully point out that this is a well-defined class of drugs with non-limiting examples well known in the art. *See, e.g.*, Gale and Lin; *see also* V. Bichko *et al.*, "Valopictabine (NM283) is Fully Active Against Known HCV Protease Resistance Mutations *In Vitro*," 42nd Annual Meeting of the European Association for the Study of the Liver, Barcelona, Spain, April 11-15, 2007 ("Bichko") (copy enclosed). It is not required that the specification recite explicitly what is well known in the art to meet the enablement requirement. MPEP § 2164.03. Therefore, the specification, combined with the knowledge of the skilled artisan, enables the full breadth of the presently amended claims.

### 3. Working Examples

The Examiner alleges that the specification “fails to provide any working embodiments that meet the claimed limitations.” (Office Action, page 10). Applicants disagree. The specification discloses working examples of *in vitro* combination therapy at pages 129-131. In Example 6,  $\beta$ -D-2'-methyl cytidine, a compound recited in the claims, is shown to be effective in combination with interferon alpha-2b against BVDV, a well-know surrogate for hepatitis C virus. (Example 6, page 129). Even in unpredictable arts, a disclosure of every operable species is not required to satisfy enablement. MPEP § 2164.03. Based on the examples of the specification and the references cited above, one skilled in the art would be able to select additional combinations of 2'-branched nucleosides and drugs known in the art as inducing mutations in the hepatitis C virus without undue experimentation.

### 4. Guidance in the Specification

The Examiner alleges that the claims “do not provide any structural limitations” for drugs that induce a mutation other than a change from serine in the NS5b region. (Office Action, page 10). Applicants respectfully point out that drugs that induce mutations in a hepatitis C virus, and their structures, are well-known in the art. Moreover, the specific drugs that may be used in combination with the 2'-branched nucleosides of the instant claims are limited by their ability to induce mutations in a hepatitis C virus. Applicants respectfully point out that this is a well-defined class of drugs with non-limiting examples well known in the art. (*See, e.g.*, Gale, Lin and Bichko). Thus, one skilled in the art, reading the specification in context of what is known in the art, has adequate guidance to practice the claimed invention.

Regarding guidance for treatment of additional viruses in the *Flaviviridae* family, Applicants respectfully point out that the claims have been amended to recite hepatitis C virus as the virus to be treated.

### 5. State of the Prior Art

The Examiner alleges that the art teaches that interferon alpha-2b is “indispensable in the combination therapy for HCV infection.” (Office Action, page 11). Applicants respectfully disagree. For example, it has been shown that the combination of NM107, a 2'-branched nucleoside, and boceprevir, an HCV protease inhibitor, is effecting against drug resistant hepatitis C virus infections. D. Standring *et al.*, “Inhibitors of HCV Polymerase (NM107) and Protease (boceprevir) in Combination Show Enhanced Activity and Suppression

of Resistance in the Replicon System,” 58th Annual Meeting of the American Association for the Study of Liver Diseases, Boston, MA, November 2-6, 2007 (“Standring”) (copy enclosed). Thus, the state of the art teaches that a variety of different combinations of anti-hepatitis C drugs may be used to treat hepatitis C infections. Furthermore, those skilled in the art recognize that a correlation exists between *in vitro* inhibition of hepatitis C virus replicon RNA levels and the *in vivo* treatment of hepatitis C virus infections. (See, e.g., Standring and Bichko). Where a particular model is recognized as correlating to a specific condition in a given art, the Examiner should accept that correlation unless the Examiner has evidence that the model does not correlate. (See MPEP § 2164.02; see also *In re Brana*, 51 F.3d 1560, 1566, 34 U.S.P.Q.2d 1436, 1441 (Fed. Cir. 1995)). Therefore, Applicants point out that the state of the art is not as unpredictable as the Examiner suggests.

#### **6. The Amount of Experimentation Needed**

The Examiner claims that it would require undue experimentation to practice the claimed invention due to alleged lack of working examples. (Office Action, page 11). Applicants disagree. Even a large amount of merely routine experimentation is not undue. See *Wands*, 8 U.S.P.Q.2d at 1404. Here, the specification discloses working examples of *in vitro* combination therapy at pages 129-131. As demonstrated above, there is sufficient predictability in the art to select further second anti-hepatitis C drugs that induce a mutation in the hepatitis C virus. Thus, Applicants submit that it would require a minimal amount of routine work to select additional antiviral drugs known to induce a mutation in the hepatitis C virus, and combine them with a 2'-branched nucleoside of the instant claims.

The Examiner cites *In re Angstadt*, 537 F.2d 498 (C.C.P.A. 1976) for the proposition that the scope of the claims must reasonably correlate to the scope of enablement provided in the specification. (Office Action, pages 11-12). Applicants respectfully point out that *Angstadt* supports the conclusion that the instant claims are enabled by the specification. In *Angstadt*, the court held that, even in the unpredictable chemical arts, section 112 does not require disclosure of every species encompassed by the claims. *Angstadt*, 537 F.2d at 502-03. Indeed, the court found that the claimed chemical process was fully enabled even though the examples disclosed represented only a small fraction of the species claimed. The same principle applies in the instant case— while the specification does not provide examples of every possible combination of 2'-branched nucleoside and a second mutation-inducing anti-hepatitis C virus drug, the examples provided and the state of the art enable the full scope of the instant claims. Therefore, considering the above factors as a whole, the scope of the

instant claims is enabled, as it would require a minimal amount of experimentation to select additional well-known antiviral drugs that induce a mutation in the hepatitis C virus, and combine them with a 2'-branched nucleoside as claimed.

#### **IV. Claims Rejections under 35 U.S.C. § 102.**

The Examiner has rejected claims 33-34, 37, 48-50, 92, 104 and 107-108 under 35 U.S.C. § 102(e) as allegedly anticipated by U.S. Patent No. 7,105,499 to Carroll *et al.* ("Carroll"). Carroll discloses certain nucleoside compounds for treating *Flaviviridae* infections. Carroll also discloses combination therapy with "one or more agents useful for treating HCV infections." (Column 32, lines 9-13). The instant claims recite, *inter alia*, a method of treating hepatitis C virus infections with a 2'-branched nucleoside, in combination with a second antiviral agent that induces a mutation other than a change from serine to a different amino acid in the conserved consensus sequence, *XXSGXXXT*, of domain B of the RNA polymerase region of the virus. Carroll does not disclose that the second agents used in combination therapy induce the claimed mutations in a hepatitis C virus. Indeed, Carroll mentions nothing about hepatitis C virus mutations.

"A claim is anticipated only if each and every element as set forth in the claim is found, either expressly or inherently described, in a single prior art reference." MPEP § 2131, quoting *Verdegaal Bros. v. Union Oil Co. of California*, 814 F.2d 628, 631, 2 U.S.P.Q.2d 1051, 1053 (Fed. Cir. 1987). Because Carroll does not disclose that the second agents used in combination therapy induce the specified mutations in a hepatitis C virus, the instant claims are not anticipated by Carroll. Therefore, Applicants respectfully request that the rejection under 35 U.S.C. § 102(e) be withdrawn.

#### **V. Claims Rejections Under 35 U.S.C. § 103.**

The Examiner has rejected claims 39-40, 89, 100-103, 105 and 106 under 35 U.S.C. § 103(a) as allegedly unpatentable over Carroll in view of "Sinko *et al.* (1998)." (Office Action, page 13). Applicants respectfully point out that the Examiner has not provided a citation to identify the "Sinko" reference. To Applicants' knowledge, the Sinko reference does not appear on the Examiner's Notice of References Cited, a copy of which was attached to the Office Action, nor in any of the Forms PTO-1449 submitted by the Applicants. Therefore, Applicants cannot respond to this rejection until the Examiner identifies the cited reference.



**CONCLUSION**

In view of the foregoing, it is submitted that this application is in condition for allowance. Favorable consideration and prompt allowance of the application are respectfully requested.

Submitted herewith are fees for an Extension of Time for three months (\$1,050.00). Please apply any other charges, or any credits, to Jones Day Deposit Account No. 503013 (ref. no. 417451-999064).

If the Examiner believes it would be useful to advance prosecution, the Examiner is invited to telephone the undersigned at (858) 314-1200.

Date: February 5, 2008

Respectfully submitted,



Dale Rieger

Reg. No. 43,045

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End Of Therapy Results		
Treatment Group	Triple	Double
48 Week HCV-RNA	46% (188/413)	48% (206/425)
Genotype 1	42% (125/299)	42% (138/306)
Stage 3-4	39% (54/140)	38% (54/141)

## Disclosures:

Frank Adams - Schering-Plough: Investigator  
 Nezam Afdhal - Schering-Plough: Investigator  
 Alamo Study Group - No relationships to disclose  
 Norma S Cantu - Schering-Plough: Investigator  
 James Cox - Schering-Plough: Investigator  
 Michael Curry - Schering-Plough: Investigator  
 Mitchell Davis - Schering-Plough: Investigator  
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**DIRECT INHIBITION OF INTRACELLULAR HEPATITIS C VIRUS REPLICATION BY RIBAVIRIN AND ITS SYNERGISTIC ACTION USED IN COMBINATION WITH INTERFERON-ALPHA.** Yoko Tanabe, Naoya Sakamoto, Nobuyuki Enomoto, Masayuki Kurosaki, Eri Ueda, Shinya Maekawa, Tuyoshu Yamashiro, Mina Nakagawa, Cheng-Hsin Chen, Nobuhiko Kanazawa, Mamoru Watanabe, Tokyo Medical and Dental University, Tokyo, Japan

**Backgrounds:** Combination therapy of interferon-alpha (IFN) and ribavirin against hepatitis C virus (HCV) infection has achieved superior clinical antiviral responses to the IFN monotherapy. However, antiviral mechanisms of ribavirin against HCV have not been well characterized yet, nor the mechanisms of the combination therapy have been understood. To explore virological basis of the effect of the combination therapy, we have analyzed effects of IFN and ribavirin, singly and in combination, on intracellular HCV replication using HCV replicon system.

**Methods:** For rapid and accurate measurement of HCV replication levels, a replicon was constructed which expressed selectable chimeric reporter protein of firefly luciferase and neomycin phosphotransferase. Replicon RNA was synthesized in-vitro, and was transfected into the Huh7 cells. After G418-selection, cell harboring continuous replicon-expression was established (Huh7/Rep-Feo). The Huh7/Rep-Feo was cultured in the presence of IFN and/or of ribavirin, and replication levels were sequentially quantified by luciferase assays. The nonstructural region (NS3-NS5B) of the replicon subgenome was amplified by nested RT-PCR with 12 partially overlapping primer sets as we have previously reported. To analyze quasispecies of the replicon, The PCR-amplified fragment of the replicon subgenome was cloned into pGEM-T vector, and 10 independent clones were sequenced.

**Results:** The replicon was highly sensitive to IFN with 50% inhibitory concentration (IC50) of 0.5 U/ml. In addition, ribavirin treatment also significantly suppressed HCV replication without significant cytotoxicity, but it did at concentrations of far above the clinically achievable levels (IC50 = 126 µM). Nucleotide diversity of the replicon sequence was significantly increased by the ribavirin treatment, suggesting induction of error prone HCV replication by ribavirin. Importantly, combinatory use of physiological concentrations of ribavirin with IFN showed strong synergistic inhibitory effects on the HCV replication.

**Conclusion:** Our results suggest that the direct inhibition of intracellular HCV replication by ribavirin and its synergistic action combined with interferon-alpha may explain the better clinical responses of the combination therapy.

## Disclosures:

Cheng-Hsin Chen - No relationships to disclose  
 Nobuyuki Enomoto - No relationships to disclose  
 Nobuhiko Kanazawa - No relationships to disclose  
 Masayuki Kurosaki - No relationships to disclose  
 Shinya Maekawa - No relationships to disclose  
 Mina Nakagawa - No relationships to disclose  
 Naoya Sakamoto - No relationships to disclose  
 Yoko Tanabe - No relationships to disclose  
 Eri Ueda - No relationships to disclose  
 Mamoru Watanabe - No relationships to disclose  
 Tuyoshu Yamashiro - No relationships to disclose

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**META-ANALYSIS OF ISDR MUTATIONS AND INTERFERON-ALPHA (IFNA) SENSITIVITY IN PATIENTS WITH CHRONIC HEPATITIS C VIRUS (HCV) GENOTYPE 1B INFECTION.** Maria E Pascu, Campus Virchow, Charite, Berlin, Germany; Peter Martus, Institute of Medical Informatics, Biometry and Epidemiology, Berlin, Germany; Marina Hoehne, Robert Koch Institut, Berlin, Germany; Bertram Wiedenmann, Uwe Hopf, Campus Virchow, Charite, Berlin, Germany; Eckart Schreier, Robert Koch Institut, Berlin, Germany; Thomas Berg, Campus Virchow, Charite, Berlin, Germany

Conflicting results were reported regarding the correlation between the number of mutations in the NS5A gene (aa 2209-2248), the interferon-sensitivity-determining-region (ISDR), and IFN effectiveness in HCV genotype 1b infected patients. The aim of this meta-analysis was to investigate the relationship between the number and pattern of mutations in the ISDR, pre-treatment viral load, geographical area and IFN response.

SR was defined as negative serum HCV-RNA for 6 months after treatment. Any other behaviour of HCV-RNA was considered nonresponse (NR). Thus, a database, comprising information on 1230 ISDR strains (952 IFN-resistant and 278 IFN-sensitive) from 28 published studies, was constructed and analysed by logistic regression analysis.

The distribution of wild (identical to HCV-J), intermediate (1-3 mutations), and mutant ( $\geq 4$  mutations) type ISDR sequences was significantly different in the Japanese as compared to the European population (44.1%, 37.6%, and 18.3% versus 24.8%, 63.4%, and 11.8%;  $p < 0.001$ ). The cumulative analysis demonstrated a clear positive correlation between SVR and type of ISDR sequence not only in the Japanese but also in the European patients. However, the SVR in Japanese patients infected with wild, intermediate, and mutant ISDR type was significantly different in Japanese in comparison to European patients (10.4%, 17.5%, and 79.2% versus 6.2%, 18.9%, and 43.5%;  $p < 0.001$ ). The likelihood of SVR with each additional mutation within the ISDR was significantly greater in Japanese in comparison to European patients (odds ratios: 1.82 versus 1.39,  $p < 0.001$ ). Mutations frequency at any of the ISDR sites didn't improved the SVR likelihood as compared to the total number of mutations per individual.

The phylogenetic tree analysis showed neither clusters of ISDR isolates from Japanese or European patients nor cluster of IFN-sensitive and IFN-resistant ISDR isolates. No major impacts on the secondary structure of the ISDR protein were observed as revealed by hydrophobicity analysis.

The SVR in Japanese patients correlated stronger with pretreatment viremia than in European patients. Thus, a pre-treatment viral load of  $> 6.6$  log copy/ml independently of ISDR type was associated with 0% SVR rate in Japanese patients and with 12.4% SVR rate in European patients. A pre-treatment viral load of  $< 6.6$  log copy/ml and infection with ISDR mutant type is associated with a SVR rate of 97.1% in Japanese patients but only 52.5% in European patients.

The prescribed cumulative IFN doses were significantly higher in Japanese in comparison to European patients (at 4 weeks:  $185 \pm 48$  MU versus  $56 \pm 29$  MU; at 24 weeks  $655 \pm 104$  MU versus  $290 \pm 87$  MU,  $p > 0.001$ ). It could not be concluded, whether - after

adjustment for the number of mutations - the different cumulative IFN dose or a specific viral or host genetic factor causes the different response rates in Europe and Japan: inclusion of IFN dose makes geographical area insignificant and vice versa.

The linkage between IFN sensitivity of HCV genotype 1b and number of ISDR mutations is indisputably in Japanese as well as in European patients. However, the IFN-sensitive ISDR phenotype is rather a quantitative than a qualitative feature. HCV genotype 1b isolates with multiple amino acids substitutions in the ISDR may represent the subtype with favourable response to the antiviral treatment.

Disclosures:

Thomas Berg - No relationships to disclose  
Marina Hoehne - No relationships to disclose  
Uwe Hopf - No relationships to disclose  
Peter Martus - No relationships to disclose  
Maria E Pascu - No relationships to disclose  
Eckart Schreier - No relationships to disclose  
Bertram Wiedenmann - No relationships to disclose

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**VX-950, A HCV PROTEASE INHIBITOR, RETAINS POTENCY AGAINST BILN-2061 RESISTANT REPLICON CELLS.** Chao Lin, Kai Lin, Cynthia A Gates, Sue Ma, Debra Brennan, John Fulghum, Hsun-Mei Hsiao, Govinda Rao, Yunyi Wei, John Alford, Robert B Perni, Ann D Kwong, Vertex Pharmaceuticals Inc., Cambridge, MA

Due to the limited efficacy of current therapies for chronic Hepatitis C virus (HCV) infected patients, more specific and potent anti-HCV drugs are needed.

We have been developing small molecule inhibitors of the HCV NS3-4A protease using a structure-based, rational drug design process. We recently selected VX-950 as a candidate for clinical development. In this report, we describe resistance studies, using an in vitro replicon system, conducted on VX-950 and BILN-2061, another HCV protease inhibitor, which was recently reported to be in clinical trials. Distinct drug-resistant mutations were identified for both protease inhibitors. Mutants that are resistant to BILN-2061 remain fully sensitive to VX-950. Characterization of enzymatic, kinetic, and anti-viral properties will be presented for mutations that confer resistance to VX-950 or to BILN-2061.

Disclosures:

John Alford - No relationships to disclose  
Debra Brennan - No relationships to disclose  
John Fulghum - No relationships to disclose  
Cynthia A Gates - No relationships to disclose  
Hsun-Mei Hsiao - No relationships to disclose  
Ann D Kwong - No relationships to disclose  
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Robert B Perni - No relationships to disclose  
Govinda Rao - No relationships to disclose  
Yunyi Wei - No relationships to disclose

# Valopicitabine (NM283) is Fully Active Against Known HCV Protease Resistance Mutations In Vitro

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**Background and aims:** Valopicitabine (NM283), an inhibitor of the HCV NS5B polymerase, is an orally bioavailable prodrug of NM107 (2-C-methylcytidine), and is in Phase II clinical development for the treatment of chronic hepatitis C. Agents that target the NS5A serine protease are also in development for HCV. A number of NS5A mutations that confer resistance to protease inhibitors have been identified *in vitro* and have emerged rapidly in clinical trials. This suggests that development of resistance to these agents may limit clinical efficacy, and combinations of HCV antivirals with different resistance profiles may maintain viral suppression more consistently. To evaluate the potential for development of such combinations, the present study assesses the antiviral activity of NM107 against major mutations that confer resistance to protease inhibitors.

**Methods:** Protease inhibitor resistance mutations, including R155Q, A156T, D168A, D168V, and D168Y, were introduced into the HCV replicon cDNA by site-directed mutagenesis. Following RNA electroporation and G418 selection, stable Huh7 cell lines harbouring mutant replicons were generated. Alternatively, mutant HCV replicons were transiently transfected into Huh7 cells that had been cured of the replicon for drug susceptibility studies. Inhibition of viral replication was measured by quantitative real-time RT-PCR as well as western blot analyses for NS5A protein. To identify NM283/NM107 resistance mutations, a Huh7 cell line harbouring NM107-resistant HCV subgenomic replicon was selected by passaging with increasing amounts of NM107.

**Results:** All tested protease inhibitor resistance mutations were fully sensitive to NM107, with  $EC_{50}$  values similar to that of the wild-type replicon. In separate experiments, sequencing of NM107-resistant HCV replicons identified a single mutation (S282T) in the highly conserved B domain of NS5B, that conferred a moderate degree of resistance to NM107 (<20-fold change in  $EC_{50}$  values).

**Conclusions:** These initial results suggest that valopicitabine (NM283) and HCV protease inhibitors have complementary cross-resistance profiles; thus combination therapies with these agents could be an advantageous strategy to suppress the emergence of resistance and maintain effective viral suppression. Additional *in vitro* drug combination studies are underway.

## INTRODUCTION

NM107, the active moiety of valopicitabine (NM283) is a novel nucleoside analogue that inhibits hepatitis C virus (HCV) *in vitro* by targeting the HCV polymerase through a mechanism consistent with chain termination.<sup>1</sup>

Valopicitabine is currently in Phase II clinical development for the treatment of chronic hepatitis C. In treatment-naïve patients with genotype-1 chronic HCV infection, combination therapy with valopicitabine/pegIFN- $\alpha$  has consistently demonstrated antiviral effect.<sup>2</sup>

- At Week 86, the reductions in serum HCV RNA from baseline were:  
- 1.1 log<sub>10</sub> for the 200 mg valopicitabine/pegIFN- $\alpha$  arm
- the proportion of patients attaining non-detectable serum HCV RNA at Week 86 for the 200 mg valopicitabine/pegIFN- $\alpha$  arm was 68%.

Future therapy of hepatitis C is likely to comprise combinations of small-molecule antiviral agents that target multiple HCV proteins, in order to maintain maximal viral suppression and minimise the emergence of resistance.

To evaluate the potential for development of such combinations, the present study assesses the antiviral activity of NM107 against major mutations that confer resistance to protease inhibitors.

## MATERIAL AND METHODS

### Generation of Stable Cell Line Harboring NM107-Resistant Replicon

GSI-1 cells ( $0.5 \times 10^6$ ) were passaged in media containing 0.5 mg/ml of G418 and 7  $\mu$ M of NM107. Cells were split when the monolayer reached 70% confluency and NM107 concentration was doubled every 10-20 days to a final concentration of 28  $\mu$ M. After 49 days in culture, RNA was extracted and the entire NS5B region sequenced.

NM107-resistant cells (D168V) were tested for resistance to NM107 by quantitative real-time RT-PCR (RT-qPCR).

### Generation of Stable Cell Lines Harboring Protease-Resistant Replicons

Protease resistance mutations (Figure 1) were introduced into the replicon by site-directed mutagenesis (Stratagene). This subgenomic replicon was derived from the Con1 genotype (bovine) and bears three adaptive mutations in NS4 and NS5A to allow efficient replication.<sup>3</sup>

Huh7 or GSI-1 cells that had been cured of the replicon by prolonged treatment with IFN- $\alpha$  (GSI-1, CX-107) were electroporated in OptiBuffer (ThermoFisher Scientific) with 8  $\mu$ g of *in vitro* transcribed RNA.

Cells were plated in 10 cm dishes and 24 hours later, selection began with the addition of 0.5 mg/ml of G418. The presence of the resistance mutations was confirmed by PCR of NS4 and sequencing, as described above.

### RT-qPCR

Cells were seeded in 96-well plates at 7,500 cells/well and incubated with serial dilutions of compound in media without G418 for 3 days, after which RNA was extracted using RNeasy-96-well Cell Kit (Qiagen).

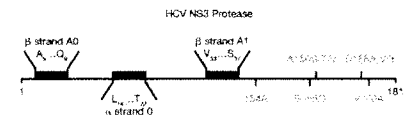
RT-qPCR was performed on a Roche LightCycler using the Sybr Green I kit (Roche) and primers specific for the HCV IRES. Forward = GGG GAG AGC CAT AGT GGT GCG GAA, reverse = GGC TAT GAG TCT GCG GGG GCG. The cycling conditions were as follows: RT-55°C 15 minutes; PCR-95°C 10 minutes; 95°C 10 minutes; 70°C 5 minutes; 72°C 10 minutes for 35 cycles. The  $EC_{50}$  values were derived by curve fit using XLfit software.

### HCV Transient Transfection Assay

The replicon cDNA was used as a template to generate *in vitro* transcribed RNA using the RNA Mega-script kit from Ambion. Eight micrograms of *in vitro* transcribed RNA was electroporated into cured GSI-1 cells.

Transfected cells were cultured for 3 days in the presence or absence of test compound, and subjected to western blot analysis. Blots were probed with a mouse anti-NS5A mAb (Novartis) and a mouse anti-1A10 mAb (Calbiochem), followed by HRP-conjugated donkey anti-mouse mAb and chemiluminescent detection. The  $EC_{50}$  values were derived by curve fit using XLfit software.

Figure 1. HCV protease-resistance mutations selected for study.



Mutation	Confers resistance to
T54A	SCH503034
R155Q	BLN2061
A156S	VX-950, SCH503034
A156T	BLN2061, VX-950, SCH503034
A156V	BLN2061, VX-950
D168A	BLN2061, Inpeptide inhibitor (IRBM)
D168V	BLN2061, Inpeptide inhibitor (IRBM)
D168Y	Tripeptide inhibitor (IRBM)
V170A	SCH503034

## RESULTS

### Generation of a Stable Cell Line Harboring NM107-Resistant Replicon

Figure 2. Culture of HCV replicon cells in the presence of up to 28  $\mu$ M of NM107 led to an S282T mutation within the B domain of NS5B, upstream of the GDD catalytic domain.

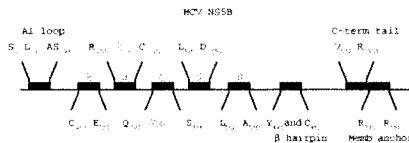


Table 1. The NM107-resistant cell line was also resistant to other 2'-nucleoside nucleosides in an RT-qPCR assay.

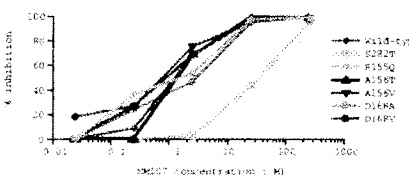
Compound	Structure	$EC_{50}$ ( $\mu$ M)	Fold change
2'-methylcytosine (NM107)		2.2 $\pm$ 0.4	24 $\pm$ 0.4
2'-methyluracil		0.2 $\pm$ 0.05	28 $\pm$ 0.7
2'-methyladenine		0.6 $\pm$ 0.03	27 $\pm$ 4.2

### Lack of Cross-Resistance in Stable HCV Replicon Cell Lines

Table 2. The D168V cell line was resistant to NM107 with a fold change consistent with previous results; the protease-resistant cell lines were resistant to a protease inhibitor (data not shown). In contrast, the protease-resistant cell lines remained susceptible to NM107, indicating no cross-resistance.

Cell line	$EC_{50}$ ( $\mu$ M)	Fold change
Wild-type	2.2 $\pm$ 0.4	-
107R	2.1 $\pm$ 0.4	15 $\pm$ 1.1
R155Q	1.2 $\pm$ 0.3	0.8 $\pm$ 0.2
A156T	1.8 $\pm$ 1.1	1.2 $\pm$ 0.7
A156S	0.5 $\pm$ 0.1	0.3 $\pm$ 0.1
D168A	1.3 $\pm$ 0.3	1.1 $\pm$ 0.4
D168V	1.5 $\pm$ 1.0	0.9 $\pm$ 0.7

Figure 3. Effect of NM107 on stable HCV replicon cell lines. Replicons resistant to NM107 and protease inhibitors, as well as the wild-type, were analysed.



### Lack of Cross Resistance in Cells Transiently Transfected with HCV Replicons

Table 3.  $EC_{50}$  values and fold change for cells transfected with the transient HCV replicon.

Replicon	$EC_{50}$ ( $\mu$ M)	Fold change
Wild-type	1.9 $\pm$ 0.3	-
T54A	3.0 $\pm$ 1.0	1.6 $\pm$ 0.02
R155Q	4.1 $\pm$ 0.6	2.1 $\pm$ 0.3
A156S	2.8 $\pm$ 0.7	1.3 $\pm$ 0.3
A156T	1.8 $\pm$ 1.3	2.5 $\pm$ 0.7
D168A	3.9 $\pm$ 1.3	2.0 $\pm$ 0.7
D168V	4.5 $\pm$ 1.9	2.2 $\pm$ 0.7
D168Y	3.2 $\pm$ 1.1	1.7 $\pm$ 0.7
V170A	2.3 $\pm$ 0.5	1.2 $\pm$ 0.3

Figure 4. Susceptibility of the transiently transfected protease-resistant replicons to NM107 in a representative western blot analysis.

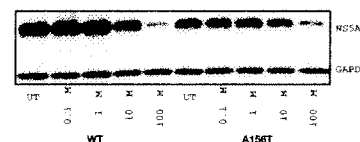
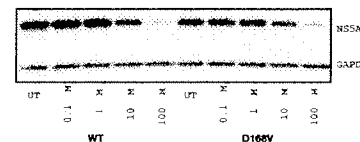
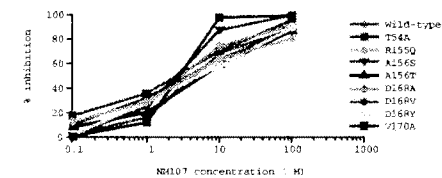


Figure 5. Effect of NM107 on the protease-resistant mutants in the HCV replicon assay (representative data shown).



## CONCLUSIONS

- A single mutation within NS5B, S282T, confers resistance to NM107 (the active moiety of valopicitabine (NM283)) and other 2'-methyl nucleoside analogues.
- Several known protease-resistance mutations are susceptible to NM107 in two different experimental HCV models, indicating a lack of cross-resistance.
- These results suggest that combination treatment of NM107 with a protease inhibitor may be an effective approach to eliminating HCV while preventing the emergence of resistance mutations.
- The clinical significance of these findings is unknown.

## ACKNOWLEDGMENTS

The authors would like to thank Dr Christoph Seeger for the GSI-1 cell line and the HCV replicon cDNA construct.

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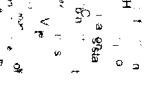
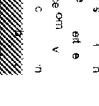
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or

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## Control of PKR Protein Kinase by Hepatitis C Virus Nonstructural 5A Protein: Molecular Mechanisms of Kinase Regulation

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**The PKR protein kinase is a critical component of the cellular antiviral and antiproliferative responses induced by interferons. Recent evidence indicates that the nonstructural 5A (NS5A) protein of hepatitis C virus (HCV) can repress PKR function in vivo, possibly allowing HCV to escape the antiviral effects of interferon. NS5A presents a unique tool by which to study the molecular mechanisms of PKR regulation in that mutations within a region of NS5A, termed the interferon sensitivity-determining region (ISDR), are associated with sensitivity of HCV to the antiviral effects of interferon. In this study, we investigated the mechanisms of NS5A-mediated PKR regulation and the effect of ISDR mutations on this regulatory process. We observed that the NS5A ISDR, though necessary, was not sufficient for PKR interactions; we found that an additional 26 amino acids (aa) carboxyl to the ISDR were required for NS5A-PKR complex formation. Conversely, we localized NS5A binding to within PKR aa 244 to 296, recently recognized as a PKR dimerization domain. Consistent with this observation, we found that NS5A from interferon-resistant HCV genotype 1b disrupted kinase dimerization in vivo. NS5A-mediated disruption of PKR dimerization resulted in repression of PKR function and inhibition of PKR-mediated eIF-2 $\alpha$  phosphorylation. Introduction of multiple ISDR mutations abrogated the ability of NS5A to bind to PKR in mammalian cells and to inhibit PKR in a yeast functional assay. These results indicate that mutations within the PKR-binding region of NS5A, including those within the ISDR, can disrupt the NS5A-PKR interaction, possibly rendering HCV sensitive to the antiviral effects of interferon. We propose a model of PKR regulation by NS5A which may have implications for therapeutic strategies against HCV.**

The interferon (IFN)-induced double-stranded RNA (dsRNA)-activated protein kinase, PKR (52), is a key component of the antiviral and antiproliferative effects of interferon (reviewed in reference 13). As a member of the IFN-induced gene family, PKR is transcriptionally activated from a low level of expression upon cellular exposure to IFN (52). Activation of PKR catalytic function proceeds through a process of dsRNA binding, dimerization, and autophosphorylation (reviewed by Clemens and Elia [13]). Tight regulation of PKR is essential for controlling the function of PKR substrates, the best characterized of which is the protein synthesis initiation factor 2,  $\alpha$  subunit (eIF-2 $\alpha$ ). PKR phosphorylates serine 51 of eIF-2 $\alpha$ , leading to limitations in functional eIF-2, a concomitant inhibition of mRNA translation initiation, and repression of cell growth (13, 51). Another PKR substrate is I $\kappa$ B, the inhibitor of nuclear factor kappa B (NF- $\kappa$ B) (43). By phosphorylating I $\kappa$ B, PKR functions within dsRNA- and IFN-signaling pathways to induce NF- $\kappa$ B-dependent transcription (44; reviewed in reference 77). PKR may also be required for IFN- $\gamma$  signaling processes (44) and is a key mediator of stress-induced apoptosis (18). Constitutive repression of PKR induces malignant transformation of mammalian cells (3, 42), thus identifying PKR as a potential tumor suppressor (4). Tumor suppressor function has been attributed to PKR-dependent eIF-2 $\alpha$

phosphorylation (54), though the other roles played by PKR may contribute to cell growth regulation as well (59).

PKR is best understood for its role in the IFN-induced cellular antiviral response (for reviews of the IFN response, see references 68 and 69). Within the IFN response, PKR-mediated phosphorylation of eIF-2 $\alpha$  provides a key antiviral function by phosphorylating eIF-2 $\alpha$  to block protein synthesis and thereby inhibit viral replication (reviewed in reference 37). To facilitate replication and avoid the antiviral effects of IFN, eukaryotic viruses have evolved a diverse repertoire of mechanisms to repress PKR function during infection (24). We have recently determined that hepatitis C virus (HCV), a member of the *Flaviviridae* (31, 70), encodes a mechanism to repress PKR. The ability of HCV to regulate PKR lies within the viral nonstructural 5A (NS5A) protein, which binds to a distinct region of PKR to repress kinase function (27). HCV is of particular interest due to the emergence of a global HCV epidemic comprising approximately 2% of the world population.

To date, type I IFN remains the only approved anti-HCV therapeutic agent, but it is effective in only 20% of HCV-infected individuals (1, 23, 49). HCV infection is characterized by progressive liver pathology, often developing into a chronic disease state, perhaps due in part to the high number of IFN-resistant viral quasispecies within the infected population (17, 71). Problematically, chronic HCV infection has been epidemiologically linked to the development of hepatocellular carcinoma and is currently the leading indicator for adult liver transplants in the United States (20). A goal of the present study was to define the molecular mechanism which underlies

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TABLE 1. Sequences of PCR NS5A oligonucleotides<sup>a</sup>

Construct	Sense <sup>b</sup>	Antisense	nt
NS5A 1b-wt	5' TAAGCTTATGGGC	5' CAAGCTTGGATCC	6260-7598
1973-2419	TCCGGCTCGTGGCT	TTAGGACATTGAGC	
NS5A	5' CATATGGGCTCCGG	5' GTCCACCGCAGACA	6260-6965
1973-2208	CTCGTGGCTA	ACTGGCTAGCTGA	
NS5A	5' GAATTCCTTCCTT	5' GTCCACCTCCGCCG	6966-7165
2209-2274	GAAGGCAACATGC	GAACGGATAC	
NS5A	5' CCTTCCATGGCCCA	5' ATCGGATCCTTATA	6877-7094
2180-2251	CATTACAGCAGAGACG	CCACCTTATTCTCTGA	

<sup>a</sup> Amino acid and nucleotide (nt) positions correspond to the PCR-amplified region. Numbering is based on the prototypic HCV-J sequence (36).

<sup>b</sup> Cloning restriction sites (described in text) are underlined.

the ability of HCV to evade the antiviral effects of IFN and induce disease.

Most relevant are the observations that sequence variation from the prototypic IFN-resistant HCV J strain (36) within the NS5A protein of the HCV polyprotein cleavage product has been associated with sensitivity to IFN in Japanese HCV genotype 1b (HCV-1b) subtypes (21, 22, 45). Viral isolates with multiple amino acid substitutions within a region of NS5A, termed the IFN sensitivity-determining region (ISDR; amino acids [aa] 2209 to 2248), were eliminated from HCV-infected patients during IFN therapy, while those exhibiting the prototypic ISDR sequence were IFN resistant, persisting at therapy cessation. We have recently demonstrated that NS5A from IFN-resistant strains of HCV-1a and -1b can physically bind PKR by an ISDR-dependent mechanism to inhibit kinase function, implicating NS5A as a mediator of the IFN-resistant HCV phenotype (27). We hypothesized that mutations within the ISDR may similarly disrupt NS5A function to render HCV sensitive to the PKR-mediated antiviral effects of IFN. In this study we conducted a detailed molecular analysis of PKR regulation by ISDR sequence variants of NS5A previously described for IFN-resistant and sensitive clinical isolates of HCV-1b (21). We show that NS5A from IFN-resistant HCV disrupts a critical step of PKR activation, resulting in repression of PKR function and a block in eIF-2 $\alpha$  phosphorylation. Mutations in the PKR-binding domain of NS5A, localized to within the ISDR, abrogated the PKR-regulatory function of NS5A. Taken together, these results suggest a molecular mechanism for IFN sensitivity of HCV that is defined, at least in part, by the sequence of the PKR-binding domain of NS5A.

#### MATERIALS AND METHODS

**Plasmid construction and site-directed mutagenesis.** Plasmids pGBT10 and pGAD425 encode the GAL4 DNA-binding domain (BD) and transcription activation domain (AD), respectively (25). pAD-PKR K296R, pAD-PKR 244-551, and pAD-PKR 244-296 encode the indicated AD-PKR fusion constructs and have been described previously (25). All NS5A 1b expression constructs were generated from pAD-NS5A, which harbors wild-type (wt) NS5A from a clinical isolate of IFN-resistant HCV-1b (NS5A 1b-wt) (27). To facilitate yeast two-hybrid protein interaction analysis, pAD-NS5A was cleaved with restriction enzymes *NdeI* and *BamHI* and the 1.4-kb insert encoding full-length NS5A (aa 1973 to 2419) was cloned into the corresponding sites of pGBT10 to yield

pBD-NS5A 1b-wt. pBD-NS5A 1973-2361 encodes a BD fusion of NS5A aa 1973 to 2361 and was generated by subcloning the *NdeI/SalI* insert from pBD-NS5A 1b-wt into the corresponding sites of pGBT10. pBD-NS5A 2120-2274 encodes a BD fusion of NS5A aa 2120 to 2274 constructed by ligating the internal 462-bp *EcoRI/BsrYI* fragment from pBD-NS5A 1b-wt into the *EcoRI/BamHI* sites of pGBT10. pBD-NS5A 1973-2208, pBD-NS5A 2209-2274, and pBD-NS5A 2180-2251 encode BD fusions of NS5A aa 1973 to 2208, 2209 to 2274, and 2180 to 2251, respectively, and were generated by PCR amplification of the corresponding pBD-NS5A 1b-wt coding region, using the restriction site-linked oligonucleotide primer pairs shown in Table 1. PCR products were directly cloned into pCR2.1 (Invitrogen) as described by the plasmid manufacturer. PCR products encoding NS5A aa 1973 to 2208 and 2209 to 2274 were released from pCR2.1 by digestion with restriction enzymes *NdeI/SalI* and *EcoRI/SalI*, respectively. The resultant insert DNA was ligated into the appropriate sites of pGBT10 and pGBT9 (Clontech) to yield pBD-NS5A 1973-2208 and pBD-NS5A 2209-2274. The PCR product encoding NS5A aa 2180 to 2251 was released from pCR2.1 by *NcoI/BamHI* digestion, and the resultant 213-bp fragment was ligated into the identical sites of pAS2-1 (Clontech) to yield pBD-NS5A 2180-2251. pBD- $\Delta$ ISDR encodes an ISDR deletion mutant of NS5A from IFN-resistant HCV-1a (27).

We used site-directed mutagenesis (Chameleon double-stranded site-directed mutagenesis kit; Stratagene) to introduce ISDR mutations corresponding to IFN-sensitive strains of HCV-1b into pBD-NS5A 1b-wt. Mutagenesis reactions were carried out as described by the manufacturer, using the mutagenic primers shown in Table 2. Template DNA was denatured by incubation at 100°C for 5 min, followed by annealing of the indicated mutagenic primer and the *SacI*-to-*SnaI* selection primer 5' GTGACTGGTGAGGCCTCAACCAAGTC (*SnaI* restriction site underlined). T7 DNA polymerase-primer extension products were ligated and selected for the primer-encoded mutation(s) by digestion with restriction enzyme *SacI* and subsequent transformation into *Escherichia coli* X1-mutS. By this method, we constructed a set of isogenic NS5A constructs, identical to NS5A 1b-wt except for the defined mutations introduced into the ISDR (Table 3). pBD-NS5A 1b-2 and pBD-NS5A 1b-4 were generated directly from pBD-NS5A 1b-wt and encode a single (A2224V) or multiple (P2209L, T2214A, and T2217G) ISDR amino acid mutations, respectively (Table 3). pBD-NS5A-5, encoding the ISDR mutations P2209L, T2214A, T2217G, and A2224V, was produced by introducing an A2224V mutation into pBD-NS5A-4.

For expression of NS5A in *Saccharomyces cerevisiae*, the entire 1.4-kb insert of pBD-NS5A 1b-wt was amplified by PCR using the restriction enzyme-linked oligonucleotides shown in Table 1. PCR products were cloned into the *SrfI* site of pCR-Script (Stratagene) and released from the resultant plasmid by *HindIII* digestion. The gel-purified insert DNA was cloned into the *HindIII* site of pYES2 (Invitrogen) to yield pYES-NS5A 1b-wt expressing NS5A under control of the galactose-inducible *GAL1* promoter. Construction of pYES-NS5A 1b-2, pYES-NS5A 1b-4 and pYES-NS5A 1b-5 was facilitated by replacing the internal 1.1-kb *SacII/SalI* fragment of pYES-NS5A 1b-wt with the internal *SacII/SalI* fragment from the corresponding pBD constructs. pYex-PKR $\Delta$ 295-300 was generously provided by P. Romano (Small Molecule Therapeutics, Inc.).

For expression of NS5A in mammalian cells, the entire 1.4-kb NS5A coding region of pYES-NS5A 1b-wt and pYES-NS5A 1b-5 was released by *HindIII* digestion and cloned into the *HindIII* site of pFLAG-CMV2 (Eastman Kodak

TABLE 2. Sequences of mutagenic NS5A oligonucleotides

Construct	Mutagenic primer <sup>a</sup>	Mutation(s)
NS5A 1b-2	5' GACTCCCCAGATGTTGACCTCATC	A2224V
NS5A 1b-5 <sup>b</sup>		
NS5A 1b-4	5' TTGTCTGCGCTTTCCTTGAAGGCAGCATGCACTGGCCGTCACGAC	P2209L, T2214A, T2217G

<sup>a</sup> Underlined codons correspond to the indicated amino acid mutations.

<sup>b</sup> See Materials and Methods for details on the construction of this construct.

TABLE 3. Sequences of the PKR-binding region and ISDR, with the corresponding IFN sensitivities of NS5A expression constructs

Name <sup>a</sup>	PKR-binding region (aa 2209–2274) <sup>b</sup>	IFN response <sup>c</sup>	Reference(s)
1b-pt	<b>PSLKATCTTHHSDPADLIEANLLWRQEMGGNITRVESEN</b> KVVMLDSFDPLRAEEDEREVSVAEEI	R	21
1b-wt	----- <b>R</b> -----H-----K-----P-----	R	14
1b-2	----- <b>R</b> ----- <b>V</b> -----H-----K-----P-----	R/S	21, 78
1b-4	<b>L</b> ----- <b>A</b> ----- <b>GR</b> -----H-----K-----P-----	S <sup>d</sup>	
1b-5	<b>L</b> ----- <b>A</b> ----- <b>GR</b> ----- <b>V</b> -----H-----K-----P-----	S	21
1a-wt	----- <b>AN</b> ----- <b>E</b> -----V-----V-----I-----P-----	R	27

<sup>a</sup> pt, HCV-J prototype reference sequence (GenBank accession no. D90208); wt, wild-type parental HCV-1b clone (GenBank accession no. AF034151 [NS5A coding region only]).

<sup>b</sup> The ISDR sequence is in boldface.

<sup>c</sup> R, IFN resistant; S, IFN sensitive; R/S, independently reported as IFN resistant or IFN sensitive in separate studies.

<sup>d</sup> The IFN response phenotype corresponding to 1b-4 has not been determined. However, based on published studies (21, 45), we predict this sequence to be associated with sensitivity to IFN.

Co.). The resulting plasmids, pFlagNS5A 1b-wt and pFlagNS5A 1b-5, respectively, encode full-length wt and mutant NS5A fused at the N terminus to the 8-aa FLAG epitope tag sequence (FLAG-NS5A) under control of the cytomegalovirus immediate-early promoter. pNeo-NS5A 1a-wt was constructed by cloning the *HindIII/XbaI* insert of pYES2-NS5A (27) into the corresponding site of pcDNA1Neo. pNeo-PKR K296R encodes the full-length inactive human PKR K296R mutant (5). For construction of pGST-NS5A 1b-wt, the 1.4-kb *NcoI/XhoI* insert DNA from pAD-NS5A (27) was isolated and the 3'-recessed termini were filled in with Klenow polymerase (66). The resulting blunt-ended DNA was cloned into the *SmaI* site of pGEX-2TK (Pharmacia Biotech), fusing the NS5A coding region in frame to the plasmid-encoded glutathione S-transferase (GST) protein. pGST-K3L encodes a GST fusion of the 88-aa vaccinia virus K3L protein (a very kind gift from E. Beattie, University of Washington). pGST-NS1 encodes a GST fusion of the influenza virus NS1 protein and was a generous gift from R. Krug (Sloan-Kettering). The fusion between the N-terminal 132 aa of the phage  $\lambda$  cI repressor and the catalytically inactive PKR K296R was constructed in pc1168 to yield pcI-PKR K296R as recently described (73). To avoid the cellular toxicity that is associated with wt PKR expression (5, 63), we used the full-length inactive PKR K296R mutant (5) for all PKR-protein interaction analyses. The nucleotide sequence of each new construct was verified by dideoxy nucleotide sequence analysis (Applied Biosystems).

**Cell culture and transfection.** Cos-1 cells (American Type Culture Collection) were grown in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum as described previously (74). For transient transfections, expression plasmid combinations were introduced into Cos-1 cells by the DEAE-dextran-chloroquine method exactly as described previously (74) or by a procedure using the Superfect transfection reagent as described by the manufacturer (Qiagen). Each set of transfections consisted of subconfluent 25-cm<sup>2</sup> cultures of approximately  $6 \times 10^5$  cells cotransfected with 5  $\mu$ g of each expression plasmid in the following combinations: pcDNA1Neo-pNeoPKR K296R and pNeoNS5A 1a-wt-pNeoPKR K296R, or pFlag-pNeoPKR K296R, pFlagNS5A 1b-wt-pNeoPKR K296R, or pFlagNS5A 1b-5-pNeoPKR K296R. Cells were harvested 48 h posttransfection, and extracts were processed for immunoprecipitation or immunoblot analyses as described previously (26).

**Protein analysis.** To prepare yeast extracts, cells from 20-ml liquid cultures were collected, washed once with ice-cold water, resuspended in ice-cold yeast lysis buffer (40 mM PIPES [pH 6.6], 100 mM NaCl, 1 mM dithiothreitol, 50 mM NaF, 37 mM  $\beta$ -glycerolphosphate, 120 mM ammonium sulfate, 10 mM 2-aminopurine, 15 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride), and lysed by the glass bead method as described previously (25). Cos-1 cell extracts were prepared in buffer I (50 mM KCl, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 20% glycerol, 0.5% Triton X-100, 100 U of aprotinin per ml 1 mM phenylmethylsulfonyl fluoride, 20 mM Tris [pH 7.5]) exactly as described previously (74). Extracts were clarified by 4°C centrifugation at 12,000  $\times$  g; supernatants were collected and stored at  $-70^\circ\text{C}$ . Cell extract protein concentration was determined using the Bio-Rad Bradford assay as described by the manufacturer.

Determination of protein expression was carried out by immunoblot analyses of 25 to 50  $\mu$ g of total protein from cell extracts as previously described (26). Proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes. Bound proteins were detected by probing the membranes with primary monoclonal antibodies specific to NS5A (anti-NS5A; a generous gift from T. Imagawa, Osaka University), human PKR (anti-PKR [47]; generously provided by A. Hovanessian, Pasteur Institute), FLAG epitope (anti-FLAG; Eastman Kodak), and GAL4 AD and GAL4 BD, (anti-AD and anti-BD, respectively; Clontech). Proteins were visualized by enhanced chemiluminescence and autoradiography. To control for potential errors in protein loading, blots were also probed with an actin-specific monoclonal antibody (anti-actin; ICN).

Immunoprecipitations were carried out with extracts representing  $2 \times 10^6$  transfected Cos-1 cells as previously described (26). Extracts (150  $\mu$ l) were thawed on ice and precleared by a 1-h incubation with protein G-agarose beads (Boehringer Mannheim) at 4°C. Supernatants were recovered by 4°C centrifugation

(12,000  $\times$  g) and mixed with anti-NS5A (1:500) or anti-FLAG (Eastman Kodak) M2 affinity gel in a final volume of 600  $\mu$ l of buffer I and incubated at 4°C for 2 or 16 h, respectively. Anti-NS5A immunocomplexes were recovered by an additional incubation with protein G-agarose beads equilibrated in buffer I. Immunocomplexes were washed five times with 1 ml each of ice-cold buffer I. Anti-FLAG M2 affinity gel immunocomplexes were further washed three times with cold Tris-buffered saline (50 mM Tris [pH 7.5]) and eluted by the addition of competitor FLAG peptide as described by the manufacturer (Eastman Kodak). Immunocomplexes were recovered by centrifugation, diluted in SDS sample buffer, and incubated at 100°C for 5 min. Immunoprecipitation products were resolved by electrophoresis on SDS-12% acrylamide gels and processed for immunoblot analysis as described above.

For isoelectric focusing (IEF) of eIF-2 $\alpha$ , yeast strains were grown 16 h in uracil-deficient synthetic defined medium containing 2% dextrose (SD medium), diluted to an optical density at 600 nm of 0.4 in uracil-deficient synthetic defined medium containing 2% raffinose and 10% galactose (SGAL medium), and grown for an additional 4 to 9 h at 30°C. Yeast extracts were prepared as described for immunoblot analysis. Proteins (16  $\mu$ g) were separated by vertical IEF (19) and blotted to nitrocellulose membranes. eIF-2 $\alpha$  was detected by immunoblot analysis using a rabbit polyclonal antiserum specific to yeast eIF-2 $\alpha$  (a generous gift from T. Dever, National Institutes of Health). In these experiments, an increase in the level of the less acidic, basally phosphorylated form of eIF-2 $\alpha$  indicates a concomitant decrease in the level of hyperphosphorylated eIF-2 $\alpha$ , which is phosphorylated by PKR on serine 51 (19, 63).

**Yeast methods.** Details of the yeast two-hybrid assay have been extensively described elsewhere (25, 27). This assay utilizes specific induction of a *HIS* reporter gene to support growth of *S. cerevisiae* Hf7c (Clontech) on histidine-deficient medium as an indicator of a two-hybrid protein interaction. *S. cerevisiae* Hf7c [*MATa ura3-52 his3-200 lys2-801 ade2-101 trp1-901 leu2-3,112 gal4-542 gal80-538 LYS2::GAL1-HIS3 URA3::(GAL4-17-mers)3-CYC1-lacZ*] was transformed with the indicated 2- $\mu$ m *TRP1* and *LEU2* expression plasmids harboring the corresponding GAL4 AD and BD fusion constructs. Transformed strains were plated onto SD medium lacking tryptophan and leucine (+His medium). After 3 days at 30°C, strains were streaked onto SD medium lacking tryptophan, leucine, and histidine (−His medium) and incubated for 3 to 6 days at 30°C. The resultant histidine-depleted colonies were replica streaked onto +His and −His media and incubated for 3 to 5 days at 30°C. Specific growth on −His medium was scored as positive for a two-hybrid protein interaction.

For determination of PKR and NS5A function *in vivo*, wt or mutant NS5A *URA3* expression plasmids were transformed in *S. cerevisiae* RY1-1 [*MATa ura3-52 leu2-3 leu2-112 gen2 $\Delta$  trp1- $\Delta$ 63 LEU2::(GAL-CYC1-PKR)<sub>2</sub>*] (63). This strain lacks the yeast eIF-2 $\alpha$  kinase GCN2 and harbors two copies of wt human PKR integrated into the *LEU2* locus under control of the galactose-inducible *GAL-CYC1* hybrid promoter (10). When grown on SGAL medium, PKR is expressed and phosphorylates endogenous eIF-2 $\alpha$  on serine 51, resulting in inhibition of mRNA translation and growth suppression (19, 63). Conversely, coexpression of wt NS5A represses these toxic effects associated with PKR function in this system, allowing strains coexpressing functional NS5A to grow on SGAL medium (27). RY1-1 strains harboring the indicated NS5A expression constructs were plated onto noninducing uracil-deficient SD medium and incubated at 30°C for 3 days. Single colonies were picked and cultured for 16 h at 30°C in uracil-deficient liquid SD medium. Aliquots of each culture were normalized to an optical density at 600 nm of 0.2 and serially diluted in 10-fold increments with sterile H<sub>2</sub>O. Then 2  $\mu$ l of each dilution was applied in replicate onto uracil-deficient SD and SGAL media and incubated for 3 to 6 days at 30°C. Strains were scored for high-dilution growth on SGAL medium, which is indicative of NS5A-mediated repression of PKR (27).

**Dimerization disruption assay.** The assay for dimerization disruption has been previously described (32, 33, 73). This assay utilizes sensitivity to phage  $\lambda$ -mediated cell lysis as an indicator of the dimerization state of a cI-PKR K296R fusion protein expressed from pcI-PKR K296R in *E. coli*. pcI-PKR K296R replicates under control of the p15A origin of replication and is thus a low-copy replicon



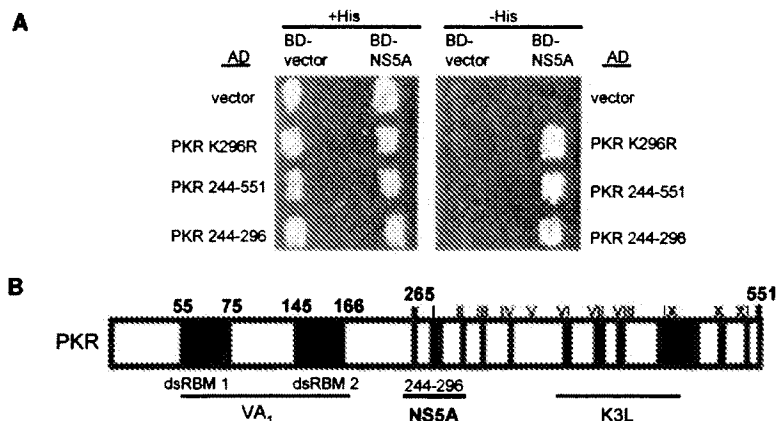


FIG. 1. NS5A-binding domain of PKR. (A) Hf7c yeast strains harboring the indicated AD and BD expression constructs were replica printed onto +His (left) and -His (right) media, incubated at 30°C for 4 days, and assayed for growth. Expression of AD-PKR and BD-NS5A 1b-wt constructs was confirmed by immunoblot analysis (not shown). Strains which grew on -His medium were scored positive for a two-hybrid protein interaction. (B) Structural representation of PKR. The positions of the two dsRNA-binding motifs (dsRBM 1 and 2) and the 11 protein kinase catalytic domain conservation regions (roman numerals) (28) are indicated in black. The regions of PKR which mediate interaction with the virus-encoded inhibitors adenovirus VA<sub>1</sub> RNA (38), vaccinia virus K3L (16, 25), and HCV NS5A proteins (reference 27 and this study) are underlined.

compatible with plasmids that contain the ColE1 origin of replication, including the pGEX series of vectors (72). *E. coli* AG1688 (33) coexpressing cI-PKR K296R and the indicated GST fusion protein was assessed for resistance to cell lysis mediated by the phage  $\lambda$  cI deletion mutant  $\lambda$ KH54 (33). *E. coli* AG1688 was grown to mid-log phase in liquid cultures consisting of Luria broth supplemented with 10 mM MgSO<sub>4</sub> and 0.2% maltose. Bacteria (2.5- $\mu$ l aliquots) were mixed with an equal volume of serial 10-fold dilutions of  $\lambda$ KH54 containing 10<sup>2</sup> to 10<sup>6</sup> PFU each. The bacterium-phage mixture was applied to antibiotic-agar plates containing 0.1 mM isopropylthio- $\beta$ -D-galactoside to induce expression of the plasmid-encoded fusion proteins. Plates were air dried, incubated 16 h at 37°C, and scored for resistance to  $\lambda$ KH54-mediated cell lysis, which is an indicator for in vivo formation of functional cI-PKR K296R homodimers. The expression of each plasmid-encoded fusion protein was verified by immunoblot analysis (data not shown).

## RESULTS

**Mechanism of PKR regulation by NS5A: disruption of protein kinase dimerization.** We previously determined that NS5A from IFN-resistant strains of HCV binds to PKR to repress kinase function (27). NS5A binding was mapped to within a broad region of the PKR catalytic domain defined by PKR aa 244 to 366. To better understand the molecular mechanism(s) of NS5A-mediated regulation of PKR, we identified a minimal NS5A-binding domain on the protein kinase. We used yeast two-hybrid analysis to examine the interaction between wt NS5A, isolated from an IFN-resistant strain of HCV-1b (27) fused to the GAL4 BD (BD-NS5A 1b-wt), and deletion mutants of PKR fused to the GAL4 AD. A two-hybrid protein interaction was confirmed by growth on -His medium (which is due to activation of the Hf7c *HIS* reporter [6]). We determined that each construct was efficiently expressed in the corresponding strains (data not shown). Hf7c yeast strains coexpressing BD-NS5A 1b-wt with AD-PKR (PKR K296R) or the AD-PKR deletion construct PKR 244-551 or PKR 244-296 all exhibited growth on -His medium, demonstrating a two-hybrid protein interaction within these strains (Fig. 1). These results define an NS5A-binding domain in PKR to within the 52-aa sequence defined by PKR aa 244 to 296. However, we cannot rule out the possibility that NS5A targets other regions of PKR. Importantly, the sequence defined by PKR aa 244 to 296 has recently been identified as a critical PKR dimerization domain (73).

We reasoned that NS5A may inhibit PKR by disrupting the PKR dimerization process. We therefore tested the ability of

NS5A 1b-wt to interfere with PKR dimerization in vivo, using a phage  $\lambda$ -based genetic assay in *E. coli*. In this assay, dimerization proteins, which are fused in frame to the DNA-binding domain of the phage  $\lambda$  cI repressor, mediate dimerization of the cI DNA-binding domain, which is required for binding to the  $\lambda$  promoter (32). When expressed in *E. coli*, the hybrid cI repressor mediates resistance to cell lysis induced by a cI deletion mutant of  $\lambda$  phage ( $\lambda$ KH54 [33]) by dimerizing and binding to the  $\lambda$  promoter. This results in repression of phage gene expression within  $\lambda$ KH52-infected *E. coli* that express the hybrid cI repressor. Conversely, coexpression of a dimerization inhibitor in this system releases  $\lambda$  gene repression through the disruption of cI dimers, resulting in *E. coli* lysis. Expression of full-length inactive PKR K296R fused at the N terminus to the cI DNA-binding domain (cI-PKR) was sufficient to repress  $\lambda$  gene expression upon  $\lambda$ KH54 infection in *E. coli* (Fig. 2). Coexpression of GST had no effect on cI-PKR-mediated  $\lambda$  gene repression, as resistance to cell lysis was observed even after exposure to high concentrations of phage. Thus, the PKR component of cI-PKR facilitates protein dimerization and  $\lambda$  gene repression in vivo. Resistance to  $\lambda$ KH54-mediated cell lysis was reduced approximately 1,000-fold in *E. coli* coexpressing cI-PKR with GST-NS5A (compare lanes 1 and 2 in Fig. 2), indicating that NS5A was disrupting the cI-PKR dimerization process. This effect of GST-NS5A 1b-wt was specific to cI-PKR, as cells coexpressing GST-NS5A 1b-wt with the dimerization control fusion protein, cI-Rop, repressed  $\lambda$  gene expression and  $\lambda$ KH54-mediated cell lysis even after exposure to high phage concentrations (lane 5). Importantly, GST constructs encoding other viral inhibitors of PKR, including vaccinia virus K3L (GST-K3L; lane 3) and influenza virus NS1 (GST-NS1; lane 4), which target the PKR-substrate (16, 25) and PKR-dsRNA (38) interactions, respectively, did not affect the ability of *E. coli* to resist  $\lambda$ KH54-mediated cell lysis when coexpressed with cI-PKR. Thus, NS5A specifically disrupts the PKR dimerization process in vivo.

**Identification of the PKR-interacting domain of NS5A: the ISDR is necessary but not sufficient for NS5A-PKR complex formation.** We previously demonstrated that the ISDR of NS5A was necessary for both interaction with and repression of PKR (27). We therefore conducted a detailed structural analysis of the NS5A-PKR interaction, using the yeast two-

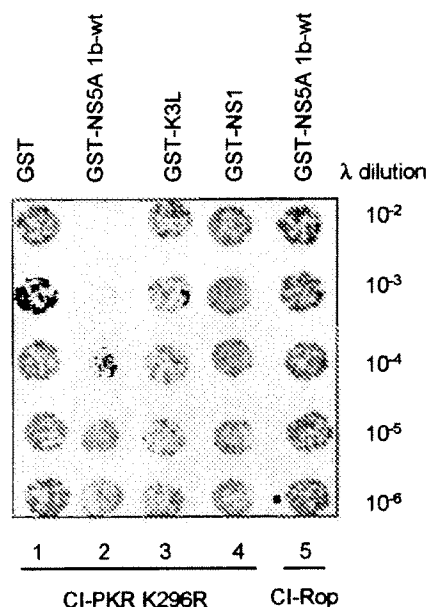


FIG. 2. NS5A disrupts PKR dimerization. *E. coli* AG1688 cells were cotransformed with expression plasmid combinations encoding CI-PKR K296R and GST (column 1), GST-NS5A 1b-wt (column 2), GST-K3L (column 3), or GST-NS1 (column 4), mixed with the indicated dilution of  $\lambda$ KH54 phage, and spotted onto plates containing agar medium. Plates were incubated and visually scored for colony formation (dark spots) as described in Materials and Methods. Column 5 contains *E. coli* harboring CI-Rop and GST-NS5A 1b-wt expression plasmids (control). CI fusion protein dimerization is indicated by colony formation. Shown is a photograph of a plate from a representative experiment.

hybrid assay to determine the role of the ISDR in this interaction. *TRP1* plasmids encoding full-length or deletion mutants of BD-NS5A 1b-wt (Fig. 3A; Table 3) were introduced into yeast strain Hf7c harboring a *LEU2* plasmid encoding AD-PKR. All constructs were expressed in cotransformed strains (Fig. 3C). Strains harboring each BD-NS5A construct and AD-PKR or AD vector (interaction-negative control [not shown]) all grew on this medium. Interaction-negative control strains failed to grow on  $-$ His medium, demonstrating specificity for the described interactions (data not shown). Using an identical assay, we previously determined that the NS5A ISDR was required for interaction with PKR (27). Importantly, we found that an ISDR-inclusive 66-aa region of NS5A was required for complex formation with PKR in vivo (Fig. 3). The NS5A N-terminal region alone (aa 1973 to 2208) was not sufficient to interact with AD-PKR, as determined by the inability of strains harboring this construct to grow on  $-$ His medium (Fig. 3B). Moreover, we found that the ISDR-inclusive construct encoding NS5A aa 2180 to 2251 did not support growth on  $-$ His medium when expressed with AD-PKR. It is important to note that this construct was expressed to levels equal to or higher than those of the overlapping construct, BD-NS5A 2120-2274, which scored positive for PKR interaction in our assay (Fig. 3B and C). Those strains harboring BD-NS5A construct 1973-2419 or 1973-2361 exhibited growth on  $-$ His medium, implying a two-hybrid protein interaction. By these analyses, we determined that the PKR-binding region of NS5A mapped to within a 66-aa region comprising the ISDR and the adjacent C-terminal 26 aa (Fig. 3A). Thus, the ISDR was necessary but not sufficient for the NS5A-PKR interaction. Examination of the amino acid sequence within the PKR-binding domain of NS5A revealed that this region is

highly conserved between our NS5A 1b-wt construct and the prototypic HCV-J sequence (Table 3). However, the NS5A-PKR interaction appears to tolerate nonconservative amino acid substitutions within this region (27).

**The NS5A-PKR interaction is dependent on the sequence of the PKR-binding domain of NS5A and is disrupted by ISDR mutations.** ISDR mutations which correlate with IFN sensitivity of HCV localize to within the PKR-binding domain of NS5A (Table 3 and references 20 and 21). We used the yeast two-hybrid assay to examine the effects, if any, that defined ISDR mutations had on in vivo complex formation between NS5A and PKR. We first prepared a series of NS5A expression plasmids encoding wt and ISDR variants of NS5A corresponding to IFN-resistant and -sensitive strains of HCV, respectively. Rather than randomly assigning ISDR mutations, we used site-directed mutagenesis to construct ISDR variants of NS5A based on defined mutations previously identified within clinical isolates of IFN-sensitive strains of HCV (21). These mutations (Table 3) were introduced into the ISDR of NS5A 1b-wt, previously isolated from IFN-resistant HCV (27). We thus generated the isogenic NS5A constructs 1b-2, 1b-4, and 1b-5, which contained two, four, and five, respectively, amino acid changes from the prototype ISDR sequence from IFN-resistant HCV. These constructs were identical to NS5A 1b-wt except for defined mutations within the ISDR and thus allowed us to determine the effects of specific ISDR mutations on HCV-1b NS5A function. Table 3 compares the ISDR sequence and IFN response of the prototype IFN-resistant HCV J strain (36) with the ISDR sequence and response to IFN determined for the corresponding viral isolate from each wt and mutant NS5A construct. The IFN sensitivity corresponding to the ISDR sequence of construct 1b-4 has not been described, although based on previous work (21), we propose that such a sequence may correlate with an IFN-sensitive phenotype.

BD-NS5A constructs encoding full-length 1b-wt NS5A and isogenic ISDR variants (Table 3) were transformed into Hf7c yeast cells harboring plasmid-encoded AD-PKR or the AD control vector. As additional controls, we included a parallel assessment of strains harboring plasmids encoding AD-PKR and the HCV-1a NS5A construct BD-1a-wt (positive control) or BD- $\Delta$ ISDR (negative control), the latter lacking the complete ISDR of the corresponding 1a-wt construct (27). The resulting yeast strains were replica printed onto  $+$ His and  $-$ His medium. All strains grew on  $+$ His medium (Fig. 4A). However, only those strains expressing BD-NS5A construct 1b-wt, 1b-2, or the 1a-wt control exhibited growth on  $-$ His medium, indicating that these constructs could bind PKR in vivo. Similar to the  $\Delta$ ISDR control, ISDR variants of BD-NS5A, 1b-4, and 1b-5 failed to interact with AD-PKR, as strains containing these constructs failed to grow on  $-$ His medium (Fig. 4A). Immunoblot analyses demonstrated that all BD-NS5A constructs and the AD-PKR construct were efficiently expressed in cotransformed yeast (Fig. 4B). Thus, isogenic NS5A constructs differing only in the ISDR sequence differentially interacted with PKR in vivo. Introduction of a single ISDR point mutation (NS5A 1b-2) was not sufficient to abolish the NS5A-PKR interaction, while multiple ISDR mutations did abolish complex formation with PKR (Fig. 4A). The inability of NS5A to bind PKR can therefore be attributed to multiple mutations within the ISDR which, importantly, have been associated with IFN-sensitive HCV quasiespecies.

**ISDR mutations abolish NS5A function.** NS5A represses PKR function through a direct interaction with the protein kinase (27). Multiple ISDR mutations disrupt NS5A-PKR complex formation (Fig. 4). It was therefore essential to compare the abilities of wt and ISDR variants of NS5A to regulate

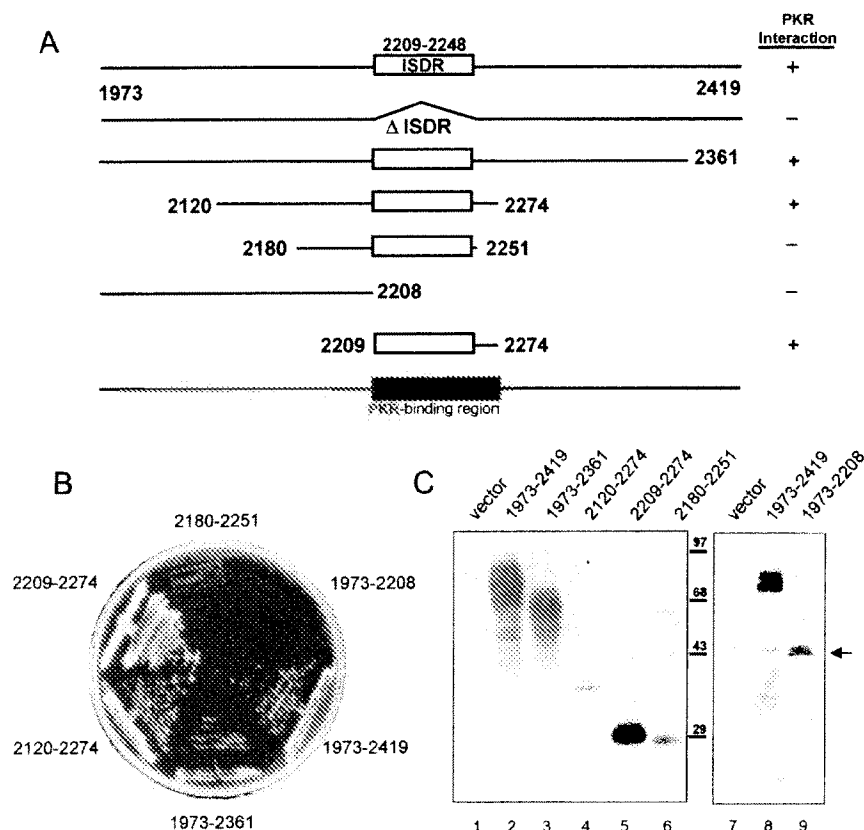


FIG. 3. PKR-binding domain of NS5A. (A) Structural representation of BD-NS5A fusion constructs. Deletion mutants were prepared from NS5A 1b-wt, except for the  $\Delta$ ISDR construct, which was prepared from NS5A 1a-wt (27). The ISDR and the PKR-binding region are shown as white and black rectangles, respectively. Terminal amino acid positions are indicated, with numbering based on the prototypic HCV-J polyprotein sequence (36). The PKR interaction of each construct (scored in panel B) is indicated at the right. (B) Yeast two-hybrid assay. Hf7c yeast strains harboring AD-PKR K296R were cotransformed with the indicated BD-NS5A deletion constructs. Strains were propagated on +His medium for 3 days (not shown), after which single colonies were streaked onto -His medium and assayed for growth. Shown is a -His plate incubated for 3 days at 30°C. Growth on -His medium is indicative of a two-hybrid protein interaction. In parallel experiments, we determined that the indicated BD-NS5A constructs did not interact with a construct encoding the GAL4 AD alone (not shown). (C) Immunoblot analysis. Extracts prepared from the yeast strains shown in panel B were separated by SDS-PAGE and subjected to immunoblot analysis using anti-NS5A (lanes 1 to 6) or anti-BD (lanes 7 to 9) monoclonal antibody. Lanes 1 and 7 contain extracts prepared from strains harboring the pGBT9 BD vector (control). Extracts are identified by the construct designation shown above the corresponding lane. BD-NS5A construct 1973-2419 was included as a positive control for the blot shown at the right. Positions of protein standards are indicated in kilodaltons. Arrow points to the protein expressed by the 1973-2208 construct.

PKR function in vivo. Expression plasmids encoding NS5A constructs 1b-wt, 1b-2, 1b-4, and 1b-5 under control of the *GAL* promoter were introduced into the *gcn2 $\Delta$*  *S. cerevisiae* strain RY1-1 (63). This strain lacks the endogenous GCN2 protein kinase and harbors two integrated copies of wt human PKR under control of a galactose-inducible *GAL-CYC* hybrid promoter (10). When placed on galactose medium, mammalian PKR is expressed and phosphorylates serine 51 on the endogenous yeast eIF-2 $\alpha$ , resulting in suppression of cell growth (63). We have demonstrated that NS5A 1a-wt can repress PKR function when coexpressed in strain RY1-1, resulting in reduced levels of eIF-2 $\alpha$  phosphorylation, enhanced protein expression, and restoration of cell growth on galactose medium (27). Strains harboring an NS5A expression plasmid or the expression plasmid alone (vector; control) grew equally well when cell equivalents were serially plated onto noninducing medium containing dextrose as the sole carbon source (Fig. 5A, left). In contrast, only the strain coexpressing NS5A 1b-wt exhibited significant growth on inducing medium (Fig. 5A, right). By this method, we determined that strains coexpressing NS5A 1b-wt with PKR exhibited greater than a 100-fold increase in plating efficiency when grown on galactose medium

compared to those strains coexpressing NS5A 1b-4 or 1b-5 and PKR. Thus, multiple ISDR mutations, corresponding to IFN-sensitive HCV, resulted in loss of the growth restoration phenotype associated with NS5A 1b-wt. In contrast, we observed only a 10-fold reduction in the plating efficiency of strains coexpressing PKR and NS5A 1b-2 (Fig. 5A). This may be due to a reduction in the PKR-binding affinity imposed by the A2224V point mutation within the NS5A 1b-2 ISDR, which does not completely abolish interaction with PKR (Fig. 4).

It is well established that inhibition of PKR results in stimulation of mRNA translation and higher levels of plasmid-encoded protein expression within yeast and mammalian cells (27, 39, 74). Such a relationship was confirmed in assays using the RY1-1 strains described above, which coexpressed PKR and ISDR variants of NS5A. Immunoblot analysis of extracts prepared from these strains revealed that PKR and the respective NS5A constructs were expressed in each strain (Fig. 5B). Importantly, this analysis revealed that the relative levels of PKR and NS5A 1b-wt were significantly increased in the corresponding strain, while levels remained unchanged among the strains harboring the comparatively nonfunctional NS5A 1b-2, 1b-4, or 1b-5. Consistent with previous results (25, 26), the

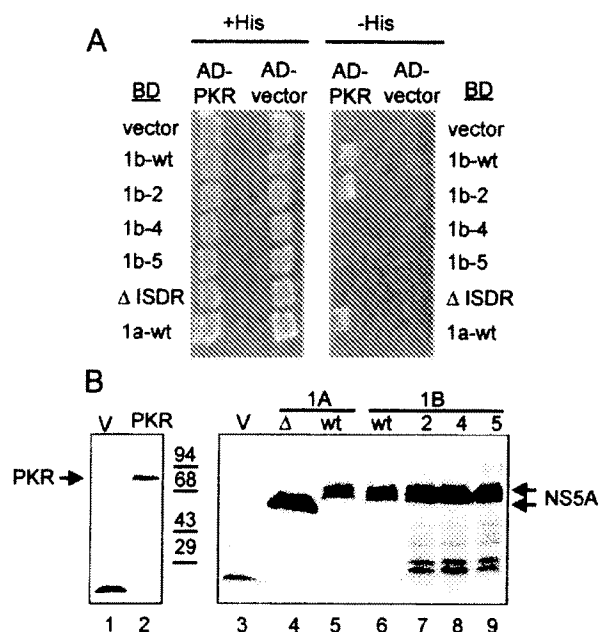


FIG. 4. Effects of ISDR mutations on the NS5A-PKR interaction. (A) Yeast two-hybrid assay. Hf7c yeast strains harboring pGAD425 encoding AD-PKR K296R (AD-PKR) or the AD alone (AD-vector) were cotransformed with pGBT9 encoding the BD alone (vector), BD-NS5A 1a-wt, BD-NS5A- $\Delta$ ISDR, or an isogenic set of BD-NS5A 1b-wt constructs possessing the ISDR sequence shown in Table 3. Strains were replica printed onto +His (left) and -His (right) media and incubated for 3 days at 30°C. Growth on -His medium is indicative of a two-hybrid protein interaction. (B) Immunoblot analysis. Extracts were prepared from the strains shown in panel A and subjected to immunoblot analysis using anti-AD (left) or anti-BD (right) monoclonal antibody. Lanes 1 and 2 show expression of the AD vector (V; control) and AD-PKR (PKR; arrow), respectively. Lanes 3 to 9 show expression of the BD vector (V; lane 3), BD-NS5A- $\Delta$ ISDR ( $\Delta$ ; lane 4), BD-NS5A 1a-wt (wt; lane 5), BD-NS5A 1b-wt (wt; lane 6), BD-NS5A 1b-2 (2; lane 7), BD-NS5A 1b-4 (4; lane 8), and BD-NS5A 1b-5 (5; lane 9). Arrows at the right indicate positions of the  $\Delta$ ISDR and full-length BD-NS5A constructs. Positions of protein standards are shown in kilodaltons.

steady-state levels of actin, which are not limiting under these experimental conditions, did not change. Importantly, we determined that all NS5A constructs were expressed to equal levels in a yeast isogenic control strain which lacks PKR (data not shown). The relative expression patterns of the NS5A constructs in RY1-1 reflected the growth properties of strains on galactose medium (compare Fig. 5A and B). These results indicate that NS5A 1b-wt repressed the translational regulatory properties of PKR in vivo, resulting in restoration of cell growth and stimulation of protein synthesis. The loss of function associated with the NS5A ISDR variants suggest that the PKR-regulatory properties of NS5A were disrupted by the introduction of mutations within the ISDR.

To directly determine the effects of ISDR mutations on the PKR-regulatory function of NS5A, we analyzed the endogenous in vivo phosphorylation state of the PKR substrate, eIF-2 $\alpha$ . Repression of PKR function in RY1-1 yeast strains results in a reduction in the level of the hyperphosphorylated form of eIF-2 $\alpha$ , phosphorylated exclusively by PKR on serine 51 (63). By using single-dimension IEF, the hyperphosphorylated form of eIF-2 $\alpha$  can be electrophoretically separated from the less acidic, hypophosphorylated form, which lacks serine 51 phosphorylation (19). Figure 5C shows an immunoblot from an IEF gel of extracts prepared from the RY1-1 strains represented in Fig. 5, which was probed with antisera specific to yeast eIF-2 $\alpha$ .

As a control, we included extracts from strains harboring either the expression plasmid devoid of insert or the transdominant-negative PKR mutant, PKR  $\Delta$ 295-300 (Fig. 5, lane 1 or 6, respectively). PKR  $\Delta$ 295-300 inhibits wt PKR when coexpressed in yeast, resulting in reduced levels of serine 51 phosphorylation and restoration of cell growth when plated on galactose medium (63). As seen in Fig. 5C, strains expressing NS5A 1b-wt or PKR  $\Delta$ 295-300 exhibited a significant reduction in the level of hyperphosphorylated eIF-2 $\alpha$ , as demonstrated by a concomitant increase in the abundance of the hypophosphorylated eIF-2 $\alpha$  isoform relative to the vector control strain (compare lanes 1 and 2). Strains expressing the isogenic NS5A variant 1b-2, 1b-4, or 1b-5 possessed predominantly the hyperphosphorylated isoform of eIF-2 $\alpha$ , similar to the vector control strain (Fig. 5C; compare lanes 3 to 5 with lane 1). The respective level of serine 51-phosphorylated eIF-2 $\alpha$  corresponded with the growth phenotype of each strain on galactose medium (Fig. 5A), where expression of NS5A 1b-wt facilitated growth on galactose and a reduction in serine 51-phosphorylation. This phenotype was reversed by the introduction of ISDR mutations into NS5A. Our results, taken together, demonstrate that ISDR mutations which correspond to IFN-sensitive

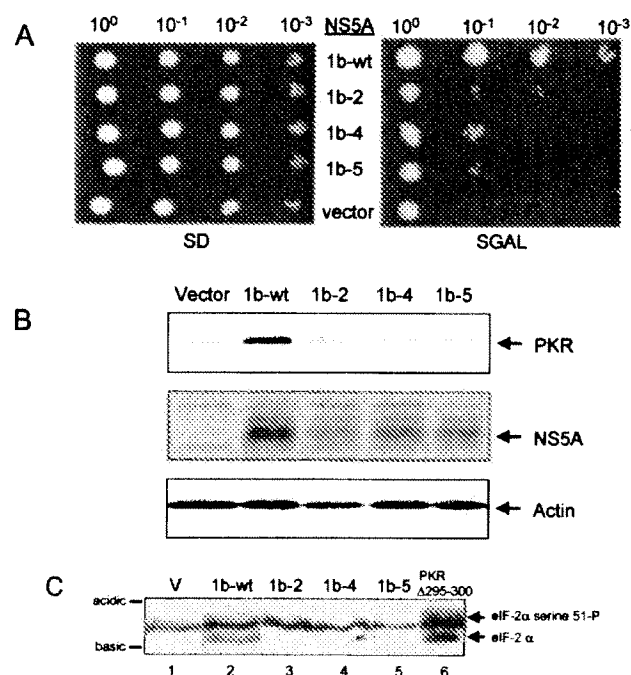


FIG. 5. ISDR mutations abolish NS5A function. (A) Yeast growth assay. Cell equivalents of RY1-1 yeast strains harboring the galactose-inducible *URA3* expression plasmid pYES-NS5A 1b-wt (1b-wt), pYES-NS5A 1b-2 (1b-2), pYES-NS5A 1b-4 (1b-4), or pYES-NS5A 1b-5 (1b-5) or the pYES control (vector) were serially diluted and spotted onto SD or SGAL medium. Panels show colony formation after 5 days growth at 30°C. (B) Immunoblot analysis of protein extracts prepared from the yeast strains shown in panel A, probed sequentially with anti-PKR, anti-NS5A, and anti-actin (control) monoclonal antibodies. Arrows at the right denote positions of PKR, NS5A, and actin. Each lane represents 50  $\mu$ g of total protein. (C) eIF-2 $\alpha$  phosphorylation. Extracts prepared from the yeast strains shown in panel A were separated by single-dimension IEF and blotted onto a nitrocellulose membrane. Detection of eIF-2 $\alpha$  was facilitated by probing the blot with anti-yeast eIF-2 $\alpha$  serum. Each lane represents 20  $\mu$ g of protein prepared from RY1-1 cells harboring pYES (V; lane 1), pYES-NS5A 1b-wt (1b-wt; lane 2), pYES-NS5A 1b-2 (1b-2; lane 3), pYES-NS5A 1b-4 (1b-4; lane 4), pYES-NS5A 1b-5 (1b-5; lane 5), or pYex-PKR  $\Delta$ 295-300 (PKR  $\Delta$ 295-300 [control]; lane 6). Arrows at the right show positions of hypophosphorylated eIF-2 $\alpha$  (lower) and hyperphosphorylated eIF-2 $\alpha$ , which is phosphorylated by PKR on serine 51. Bars at the left identify the acidic and basic ends of the blot.

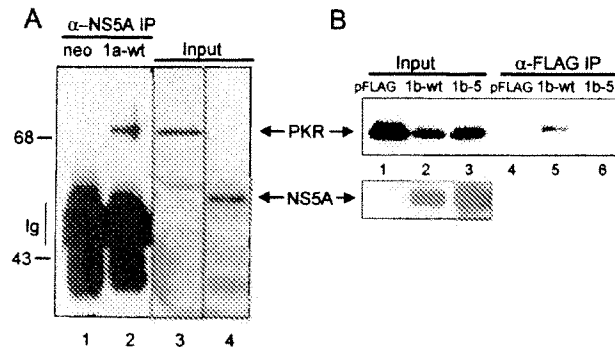


FIG. 6. ISDR mutations disrupt the NS5A-PKR association in mammalian cells. Cos-1 cells were cotransfected with cytomegalovirus expression plasmids encoding PKR K296R and NS5A or with PKR K296R and the vector control. Extracts were prepared and mixed with anti-NS5A monoclonal antibody (A) or anti-FLAG resin (B). (A) Anti-NS5A immunocomplexes prepared from extracts harboring PKR K296R with vector control (neo; lane 1) or NS5A 1a-wt (1a-wt; lane 2) and input extract (Input; lanes 3 and 4) were separated by SDS-PAGE and subjected to immunoblot analysis using anti-PKR (lanes 1 to 3) or anti-NS5A (lane 4) monoclonal antibody. Lanes 3 and 4 represent the starting material from the immunoprecipitation (IP) reaction shown in lane 2. The vertical line at left indicates the broad band corresponding to the immunoglobulin (Ig) heavy chain. Positions of protein standards are indicated in kilodaltons. (B) Immunoblot analysis of input protein (lanes 1 to 3) or protein complexes (lanes 4 to 6) recovered by mixing extracts harboring PKR K296R with vector alone (pFLAG; lanes 1 and 4), FLAG-NS5A 1b-wt (1b-wt; lanes 2 and 5), or FLAG-NS5A 1b-5 (1b-5; lanes 3 and 6) with anti-FLAG resin. Blots were probed with a monoclonal antibody specific to human PKR (top) or NS5A (bottom). Arrows point to PKR and NS5A.

HCV (Table 3) can disrupt the PKR-regulatory properties of NS5A.

**The NS5A-PKR interaction in mammalian cells is disrupted by ISDR mutations.** NS5A from IFN-resistant HCV represses the translational regulatory properties of PKR when expressed in mammalian cells (27). As suggested by the results from our yeast two-hybrid (Fig. 4) and growth (Fig. 5) assays, we predicted that the NS5A-directed repression of PKR occurring in mammalian cells was similarly mediated through a direct NS5A-PKR interaction. We therefore sought to determine if NS5A and PKR could form a stable complex in mammalian cells and what effect, if any, ISDR mutations had on complex formation. We carried out coimmunoprecipitation analyses from Cos-1 cells transiently cotransfected with plasmids encoding wt or ISDR variants of NS5A and full-length inactive human PKR. In these analyses, we used plasmids encoding NS5A from IFN-resistant HCV-1a (1a-wt), 1b-wt, and the 1b isogenic variant, 1b-5; the latter corresponding to IFN-sensitive HCV (Table 3). Immunoblot analysis of extracts prepared from cotransfected cells demonstrated that all constructs were efficiently expressed within 48 h of transfection (Fig. 6). PKR was recovered from anti-NS5A immunoprecipitates prepared from cells cotransfected with NS5A 1a-wt and from anti-FLAG immunoprecipitates prepared from cells harboring FLAG-tagged NS5A 1b-wt (Fig. 6A and B, respectively). In comparison, no PKR was detected in immunoprecipitates of FLAG-tagged NS5A 1b-5 (compare lanes 5 and 6 in Fig. 6B). Recovery of PKR was dependent on the presence of NS5A in the extract, as we failed to detect PKR in NS5A-specific immunoprecipitates prepared from extracts lacking wt NS5A (Fig. 6A and B, lanes 1 and 4, respectively). These results demonstrate that NS5A from IFN-resistant strains of HCV-1a and HCV-1b can form a stable and specific complex with PKR when expressed in mammalian cells. Consistent with our yeast two-hybrid results (Fig. 4), mutations within the ISDR which correspond to IFN-sen-

sitive HCV (Table 3) disrupted the NS5A-PKR interaction within mammalian cells. The consistency between these results and those observed in our yeast studies (Fig. 4 and 5) validates the yeast system as a viable model for the study of NS5A-PKR interaction and the effects of this interaction on PKR function.

## DISCUSSION

**Mechanism of PKR regulation: disruption of PKR dimerization by NS5A.** Molecular studies of HCV have been hampered by the lack of a suitable tissue culture system in which to support viral replication *in vitro*, though HCV has been successfully passaged in a chimpanzee model (41). To overcome this deficiency, we have developed a series of reliable yeast and mammalian cell systems to study NS5A function and PKR regulation *in vivo* (27) and used them to determine the contribution of the ISDR in NS5A-mediated regulation of PKR.

Activation of PKR is considered to be dependent on the ability of the kinase to dimerize and autophosphorylate *in trans* (2, 56, 57). This is supported by previous work which determined that active PKR resides as a dimer within mammalian cell extracts (46) and that the kinase forms a stable homodimeric complex in solution (9). Recent results indicate that PKR dimerization occurs via a mutually dependent two-step process involving dsRNA-dependent and -independent mechanisms (15, 55, 57), the latter mediated through PKR aa 244 to 296 (73). We propose a model for PKR regulation during HCV infection in which NS5A targets the PKR dimerization process by binding to within aa 244 to 296 of PKR (Fig. 1) by an ISDR-dependent mechanism (Fig. 7). Within an HCV-infected cell, viral quasispecies containing an ISDR sequence similar to the IFN-resistant HCV-J prototype (36) can persist during the course of IFN therapy through NS5A-mediated repression of PKR. In this case, the virus-encoded NS5A polypeptide cleavage product binds to PKR, targeting a kinase dimerization domain defined by PKR aa 244 to 296. Through sequences encoded within a 62-aa region spanning the ISDR and the adjacent C-terminal 26 aa (Fig. 3), NS5A disrupts the critical PKR dimerization process which is required for catalytic activity. Our data indicate that disruption of PKR dimerization results in repression of PKR function and a block in PKR-mediated eIF-2 $\alpha$  phosphorylation within the host cell. NS5A-mediated repression of PKR thereby removes the PKR-imposed block on mRNA translation and viral replication induced by cellular exposure to IFN, thus allowing HCV to resist the antiviral effects of IFN (Fig. 7, lower right).

Conversely, those viral quasispecies exhibiting ISDR sequence divergence from the prototypic HCV J strain lack the ability to disrupt the PKR dimerization process and to repress PKR function within the HCV-infected host cell (Fig. 7, lower left). ISDR variants of HCV lacking the ability to bind PKR, and/or to disrupt PKR dimerization, are thereby rendered sensitive to the antiviral effects of IFN mediated through the translational regulatory and growth-suppressive properties of PKR (Fig. 7, lower left). Moreover, NS5A function may be controlled in part by posttranslational modifications which occur within the infected host cell. NS5A resides within the cell as a phosphoprotein, present in a variety of hypo- and hyperphosphorylated states (35, 75). Our results and those of others suggest that phosphorylation of NS5A occurs by a PKR-independent process (27) and may be mediated by a CMGC-like protein kinase or a cyclic AMP-dependent protein kinase activity (34, 62). Factors which modulate such activities may thus lead to regulation of NS5A function, possibly including NS5A-mediated repression of PKR.

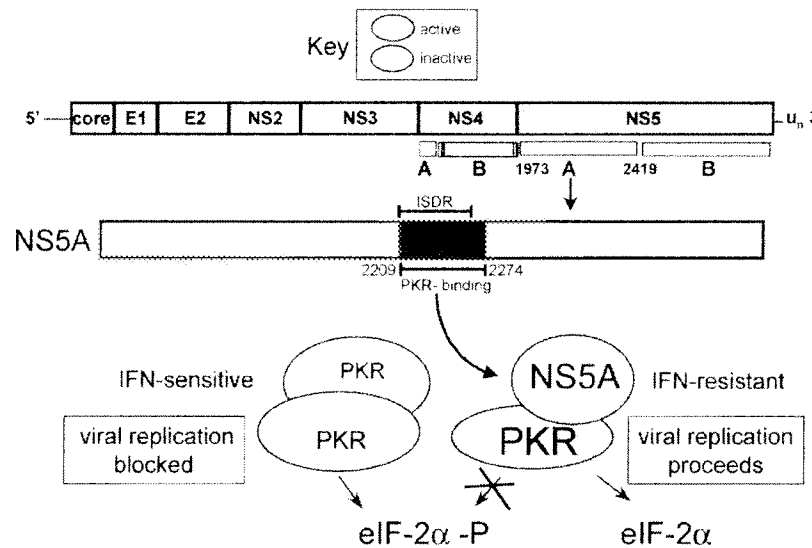


FIG. 7. Role of NS5A in PKR regulation during HCV infection. HCV sensitivity to IFN is determined, at least in part, by the structure of the PKR-binding domain (dark region) within the NS5A cleavage product of the HCV polyprotein. During HCV infection NS5A from wt, IFN-resistant strains of HCV binds PKR, disrupting the critical PKR dimerization process. Resulting PKR monomers are unable to phosphorylate eIF-2 $\alpha$ , and thus viral replication proceeds unobstructed (lower right). Mutations within the 66-aa PKR-binding region of NS5A, including the ISDR (indicated by bars), abolish the PKR-regulatory properties of HCV, rendering the virus sensitive to the antiviral actions of IFN. In this case, PKR remains active in a dimeric state and phosphorylates eIF-2 $\alpha$  to inhibit mRNA translation and viral replication (lower left).

**NS5A defines a novel class of PKR inhibitors.** PKR plays a central role within the cellular response to IFN by limiting mRNA translation and transducing IFN-mediated signals which are required for establishment of the comprehensive IFN-induced antiviral state (44, 53; reviewed in reference 67). To avoid the IFN response, many viruses encode mechanisms to disrupt PKR function which target distinct steps within the PKR maturation, regulatory, and catalytic processes. The mechanisms by which virus-directed inhibitors disrupt PKR function can be classified into five broad categories: those which (i) interfere with dsRNA-mediated PKR activation, (ii) block PKR-substrate interactions, (iii) modulate the physical levels of PKR, (iv) dephosphorylate eIF-2 $\alpha$  and/or modulate events downstream of eIF-2 $\alpha$ , or (v) disrupt PKR dimerization (reviewed in reference 24). Our results indicate that NS5A belongs to this latter group of PKR inhibitors, which also includes the cellular oncoprotein P58<sup>IPK</sup> (25, 73). Indeed, both of these inhibitors bind to sites within the same dimerization domain of PKR (Fig. 1), resulting in repression of PKR function (26, 27). Disruption of PKR dimerization was specific to NS5A and was not observed in parallel analyses of other viral inhibitors of PKR which target distinct regions of the kinase (Fig. 2). Our results support previous studies indicating that PKR dimerization is a requisite step for catalytic function (15, 57) and identify the PKR dimerization process as a key element in the regulation of PKR function.

**NS5A, PKR regulation, and IFN sensitivity: redefining the ISDR.** HCV infection is currently treated by parenteral administration of type I IFN, the only therapeutic approved for this disease (23). While high IFN response rates are associated with HCV genotypes 2 to 4, a significantly lower rate of response is observed within those individuals infected with genotype 1, suggesting that HCV encodes an IFN resistance mechanism(s) which is genotype 1 specific (76). Recent molecular epidemiological studies from Japan have identified the ISDR as a conserved region within the HCV genome of some IFN-resistant strains of HCV-1b; within Japanese patients,

mutations within the ISDR of NS5A have been associated with increased HCV sensitivity to IFN (reviewed in reference 29). We previously determined that NS5A from IFN-resistant HCV-1a and -1b strains could bind to PKR to repress kinase function *in vivo*, a process that was dependent on the NS5A ISDR (27). Using a series of NS5A ISDR variants isogenic to NS5A 1b-wt, we have now demonstrated that mutations within the ISDR can abrogate the PKR-regulatory properties of NS5A *in vivo*, which, interestingly, may be dependent on NS5A sequences both within and proximal to the ISDR. The use of isogenic ISDR variants of NS5A in these studies allows us to attribute loss of NS5A function to mutations within the ISDR. However, due to the quasispecies nature of HCV, the possibility remains that mutations in other regions of NS5A outside the ISDR also contribute to loss of NS5A function.

It has been suggested that the sequence of the ISDR in HCV isolated from patient serum may be of predictive value in predetermining the therapeutic efficacy of IFN for specific clinical cases (12, 21). Recent controversy surrounds these observations due to a lack of correlation between ISDR sequence and IFN sensitivity of HCV isolates from western Europe and the United States (30, 40, 78, 58). These difference may reflect distinct geographical features of the HCV isolates or may be due simply to variations in study parameters, IFN-dosing regimens, and/or viral genotypes examined (29). Although the present study provides strong molecular evidence to support a role for NS5A in IFN resistance, it is possible that genetic differences outside the ISDR or NS5A contribute to IFN resistance in some HCV isolates (58). Our data emphasize the need to examine additional sequences within NS5A in addition to the ISDR as originally defined. Our results argue for redefining the ISDR to include, at least, the entire PKR-binding region of NS5A (NS5A aa 2209 to 2274). Determination of sequence variations in this redefined ISDR within liver-replicating viral quasispecies may allow for the further identification of critical amino acids residues within NS5A that

are required for the repression of PKR function and resistance to the antiviral effects of IFN.

**PKR regulation and HCV pathogenesis.** In addition to its antiviral function, PKR has been identified as a critical component in dsRNA and IFN-induced signaling processes and in transcriptional regulation and as an effector of apoptosis (reviewed by Williams [77]). Moreover, several independent studies have implicated PKR as a tumor suppressor, a property that is dependent on its ability to phosphorylate eIF-2 $\alpha$  (3, 4, 7, 42, 54). Thus, NS5A-directed repression of PKR not only has implications for how HCV responds to IFN therapy but also suggests that NS5A may deregulate other PKR-dependent cellular processes. Recent results from our laboratory suggest that constitutive repression of PKR by NS5A disrupts PKR-dependent apoptosis and cell growth control (unpublished observations). Determining the long-term cellular effects from NS5A-mediated PKR repression will further our understanding of HCV pathogenesis during chronic infection.

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